MAST CELL- MEDIATED APOPTOSIS OF SMOOTH MUSCLE CELLS AND ENDOTHELIAL CELLS

Markus Leskinen

Wihuri Research Institute
Helsinki, Finland

and

University of Helsinki
Faculty of Medicine
Helsinki, Finland

ACADEMIC DISSERTATION

To be presented for public criticism, with the permission of the Faculty of Medicine of the University of Helsinki, in auditorium 1, Meilahti Hospital, Haartmaninkatu 4, on December 5th, 2003, at 12 noon.

Helsinki 2003

Supervisors:

Professor **Petri T. Kovanen** and

Docent **Ken A. Lindstedt**

Wihuri Research Institute

Helsinki, Finland

Reviewers:

Docent **Hannu Järveläinen**

Departments of Medicine and Medical Biochemistry

University of Turku

Turku, Finland

and

Professor **Veli-Pekka Lehto**

Department of Pathology

Haartman Institute, University of Helsinki

Helsinki, Finland

Official opponent:

Professor **Seppo T. Nikkari**

Department of Medical Biochemistry

University of Tampere

Tampere, Finland

ISBN 952-91-6572-2 (paperback)

ISBN 952-10-1475-X (PDF)

http://ethesis.helsinki.fi

HAKAPAINO OY 2003

To Liina

CONTENTS

ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which will be referred to in the text by their Roman numerals.

I **Leskinen M**, Wang Y, Leszczynski D, Lindstedt KA, Kovanen PT. Mast cell chymase induces apoptosis of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 2001, 21:516-522.

II Wang Y, Shiota N, **Leskinen MJ**, Lindstedt KA, Kovanen PT. Mast cell chymase inhibits smooth muscle cell growth and collagen expression in vitro: Transforming growth factor-β1-dependent and -independent effects. *Arterioscler Thromb Vasc Biol.* 2001, 21:1928-1933.

III **Leskinen MJ**, Lindstedt KA, Wang Y, Kovanen PT. Mast cell chymase induces smooth muscle cell apoptosis by a mechanism involving fibronectin degradation and disruption of focal adhesions. *Arterioscler Thromb Vasc Biol.* 2003, 23:238-243.

IV Lätti S, **Leskinen M**, Shiota N, Wang Y, Kovanen PT, Lindstedt KA. Mast cell-mediated apoptosis of endothelial cells in vitro: A paracrine mechanism involving TNF-α-mediated down-regulation of bcl-2 expression *J Cell Physiol.* 2003, 195:130-138.

V **Leskinen MJ**, Wang Y, Hakala JK, Kovanen PT, Lindstedt KA. Mast cell chymase induces smooth muscle cell apoptosis by disrupting outside-in survival signaling. Submitted, 2003

Study I has been previously published as a part of Dr. Yenfeng Wang's thesis.

ABBREVIATIONS

BSA	bovine serum albumin
BTEE	N-benzoyl-tyrosine ethyl ester
CPA	carboxypeptidase A
Da	dalton
EC(s)	endothelial cell(s)
ECM	extracellular matrix
FACS	flow cytometric analysis and cell sorting
FAK	focal adhesion kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FN	fibronectin
GAG(s)	glycosaminoglycan(s)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDL	high density lipoproteins
IFN-γ	interferon- γ
LDL	low density lipoproteins
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MMP(s)	matrix metalloproteinase(s)
NFκB	nuclear factor kappa B
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PG(s)	proteoglycan(s)
PMSF	phenylmethylsulfonyl fluoride
RP-HPLC	reverse-phase high-performance liquid chromatography
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SMC(s)	smooth muscle cell(s)
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
TRADD	tumor necrosis factor receptor-associated death domain
TRINH	soybean trypsin inhibitor
Tris	tris(hydroxymethyl)-methylamine
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling

ABSTRACT

Background- Apoptotic loss of smooth muscle cells (SMCs) and endothelial cells (ECs) may cause rupture or erosion of coronary plaques. Inflammation has been recognized as one of the key features in the pathogenesis of atherosclerosis, and has been suggested to regulate apoptosis in atherosclerotic lesions. Since mast cells form a part of the inflammatory cell infiltrate in coronary plaques, and are present in increased numbers at the vulnerable shoulder regions of human coronary atheromas, the possibility exists that mast cells may affect the stability of plaques by inducing apoptosis of SMCs and ECs.

Methods and Results- By using FACS, TUNEL, immunohistochemistry, and Western blot analysis, we found that chymase released from activated mast cells is able to induce apoptosis of SMCs *in vitro* by a mechanism involving degradation of an extracellular matrix (ECM) component, fibronectin (FN), with subsequent disruption of focal contacts on the surface of SMCs. Focal adhesion kinase (FAK), one of the key mediators of integrin-ECM interactions and cell survival, was rapidly degraded in the presence of chymase and/or FN degradation products. Loss of phosphorylated FAK (p-FAK) resulted in a rapid dephosporylation of the p-FAK-dependent downstream mediator Akt. Furthermore, following chymase treatment, the total amount of NFkappaB, a transcription factor critically involved in integrin-mediated SMC survival, was reduced and the translocation of active NFkappaB (p65) to the nucleus decreased. Loss of NFkappaB-mediated transactivation resulted in downregulation of bcl-2 mRNA expression and initiation of apoptosis. Mast cells also induced EC apoptosis by releasing tumor necrosis factor-alpha. The mechanism involves downregulation of bcl-2 and release of cytochrome c from mitochondria. Moreover, we demonstrated that mast cell chymase inhibits SMC growth and collagen expression.

Conclusions- Our results suggest that chymase and TNF-alpha -secreting mast cells, via their secretion products, mediate apoptosis of neighboring SMCs and ECs and thus may reduce the stability of coronary plaques.

INTRODUCTION

Atherosclerosis, along with infectious diseases, cancer and mental disorders, is one of the leading global health problems. The age-adjusted incidence of atherosclerosis in Finland, and the Western World in general, has steadily declined as effective treatment strategies have been developed. However, over 40% of deaths in Finland can still be attributed to atherosclerosis. This is mainly due to a demographic shift towards older population. Outside the Western World the incidence of atherosclerosis is rapidly increasing, and according to the estimates, atherosclerosis will be the leading cause of disease burden worldwide by 2020 (Murray *et al.* 1996). Our understanding of the pathogenesis of atherosclerosis has increased. Especially inflammatory mechanisms have emerged as key players in the formation of atherosclerotic plaques, suggesting that atherosclerosis is an inflammatory disease (Ross 1999; Libby 2002). The present study focuses on one component of the inflammatory system, the mast cell, and its role in the pathogenesis of atherosclerosis.

Atherosclerotic lesions develop slowly and the early lesions are usually clinically silent for decades. When clinical symptoms occur, they are predominantly caused either by narrowing of the arteries due to obstructing plaques, or by plaque erosion or rupture with subsequent local thrombus formation. The former mechanism causes stable angina pectoris and intermittent claudication. The latter causes acute clinical complications, such as unstable angina pectoris, myocardial infarction, and stroke.

Rupture of the plaque appears to depend critically on the thickness of the fibrous cap overlaying the lipid-rich core of the advanced atherosclerotic plaques (Lee *et al.* 1997): a thick cap protects the plaque, whereas a thin cap predisposes it to rupture and subsequent thrombosis

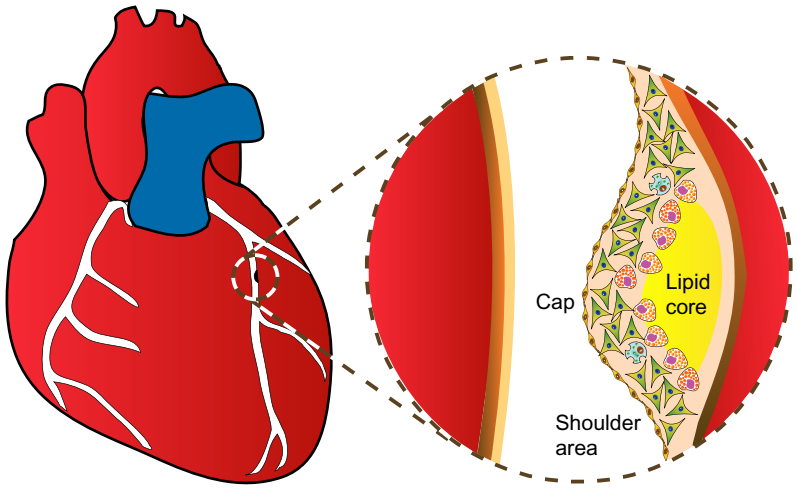

Figure 1. Schematic model of an atherosclerotic lesion

(Falk 1992). The fibrous cap consists of arterial smooth muscle cells (SMCs) and extracellular matrix (ECM) produced and maintained by the SMCs (Lee *et al.* 1997). Thus, it can be surmised, that apoptosis of SMCs leading to decreased number of SMCs and reduced production of ECM could destabilize the plaque (Björkerud *et al.* 1996; Kockx *et al.* 2000).

Apoptotic SMCs are present in fibrous caps of atherosclerotic lesions (Geng *et al.* 1995; Isner *et al.* 1995; Kockx *et al.* 1998), and they often colocalize with infiltrates of inflammatory cells (Crisby *et al.* 1997; Kockx *et al.* 1998). These include macrophages (Björkerud *et al.* 1996), T-lymphocytes (Björkerud *et al.* 1996), and mast cells (Kaartinen *et al.* 1994a; Kovanen *et al.* 1995). Moreover, in acute coronary syndromes, increased numbers of activated mast cells can be seen in the culprit lesions (Kaartinen *et al.* 1998). These observations raised the hypothesis that mast cells could participate in the regulation of apoptosis of SMCs.

Plaque erosion without rupture is another frequent cause of coronary thrombosis (Farb *et al.* 1996). One of the possible mechanisms leading to plaque erosion is apoptosis of endothelial cells (ECs) (Dimmeler *et al.* 2002). However, ECs in culture are known to be very resistant to apoptosis (Polunovsky *et al.* 1994; Bach *et al.* 1997), suggesting that special pathological conditions are required for the induction of excessive EC apoptosis in the arterial wall. Recent *in vitro* studies have shown that proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), can trigger apoptosis of ECs (Pohlman *et al.* 1989; Karsan *et al.* 1996; Wallach 1997). Since mast cells in human coronary atheromas produce and store TNF-α (Kaartinen *et al.* 1996), the possibility exists that mast cells could induce apoptosis of ECs and thus contribute to the erosion of the coronary plaques.

In the present study, we provide information on mast cell-induced apoptosis of SMCs and ECs *in vitro* and the mechanisms involved.

Review of the literature

Atherosclerosis

Atherosclerosis is a slowly progressing disease characterized by the accumulation of cells, lipids and fibrous elements in the innermost layer of the arterial wall, the intima, in large and medium-sized arteries (Stary *et al.* 1994). Atherosclerotic lesions evolve over the time from the initial, clinically silent fatty streaks into more complex lesions, resulting in clinical manifestations of the disease, such as angina pectoris, unstable angina, myocardial infarction, sudden cardiac death and stroke. The pathophysiology of atherosclerosis is a complex process involving dyslipidemia, endothelial dysfunction, and inflammation. The role of hypercholesterolemia (Ross *et al.* 1976) and endothelial dysfunction (Harker *et al.* 1981) is well established and is supported by strong experimental and clinical evidence, whereas the importance of inflammatory mechanisms in the pathophysiology of atherosclerosis has been understood only recently (Ross 1999; Libby 2002; Fan *et al.* 2003). In addition, several other factors, such as circulating smooth muscle progenitor cells (Saiura *et al.* 2001), infection (Saikku 2000) and homocysteine (Cook *et al.* 2002) have been suggested to contribute to atherosclerosis.

Early lesions

When plasma low density lipoprotein particles (LDL) are present in excess quantities, they can undergo modifications, such as oxidation, and accumulate in the intima of the arterial wall (Witztum *et al.* 1991). This, in turn can provoke an inflammatory response by inducing expression of mononuclear leukocyte adhesion molecules, such as VCAM-1, on the surface of ECs (Cybulsky *et al.* 1991; Li *et al.* 1993a; Berliner *et al.* 1995; Pentikäinen *et al.* 2000). Moreover, oxidized LDL can initiate monocyte migration into the intima by inducing production of monocyte chemotactic protein-1 (MCP-1) (Cushing *et al.* 1990). In the intima, monocytes differentiate into macrophages and begin to internalize modified LDL, thereby leading to the formation of macrophage foam cells. Subendothelial accumulation of such lipid-laden macrophages is called a fatty streak and constitutes the first visible sign of an ongoing process of atherosclerosis (Munro *et al.* 1988).

The current classification of atherosclerotic lesions, as presented by the American Heart Association, divides the lesions into six types based on their histological composition and structure (Stary *et al.* 1994; Stary *et al.* 1995). Initial intimal lesions with isolated groups of lipid-laden macrophages in the intima are defined as type I lesions. Accumulation of large numbers of lipid-laden macrophages as layers together with lipid-filled smooth muscle cells (SMCs) are classified as type II lesions (Stary *et al.* 1994). Type I lesions may occur already in human fetal aortas (Napoli *et al.* 1997), and type II lesions are present in the arteries of most children around puberty (Stary 2000), indicating the very early start of the atherosclerotic process. Early lesions are precursors of more advanced lesions, however, they never obstruct the arterial lumen or disrupt the intimal structure, and therefore are clinically silent (McGill, Jr. 1968).

Advanced lesions

Early lesions may undergo transformation into type III intermediate lesions, which contain small pools of intimal extracellular lipid, but no confluent lipid core. Confluence of these small lipid pools to form large lipid core is the first sign of clinically significant atherosclerotic lesions, defined as type IV lesions (Stary *et al.* 1995). The large lipid core of these lesions causes severe intimal disorganization, and may narrow the arterial lumen (Stary *et al.* 1995). Type IV lesions have no signs of fibrosis. It is the development of a fibrous cap, covering the lipid core, which distinguishes the type V lesions, i.e., the fibroatheroma, from the type IV lesions. Although the formation of the fibrous cap is an adaptive response, which protects the lesion from rupture, it tends to narrow the artery more than type IV lesions.

Complicated lesions

The most important mechanism of morbidity and mortality in atherosclerosis is the erosion or rupture of the atherosclerotic plaque, with ensuing thrombotic occlusion of the artery (Fuster *et al.* 1992; Falk 1992; Virmani *et al.* 2000). In autopsy studies of human coronary arteries, in 75% of cases the thrombi had formed at the site of a deep intimal tear, extending into the center of an atheromatous plaque. In 25% of cases thrombi formation was associated with superficial erosion of the fibrous cap (Davies 1990). Such disruption of the luminal surface may occur in both type IV and V lesions (Stary *et al.* 1995), resulting in the formation of the complicated type VI lesions.

Plaque rupture

The risk of plaque rupture appears to depend on plaque composition, circumferential wall stress, and characteristics of the blood flow (Falk 1992; Fuster 1994). The soft lipid core is unable to bear the mechanical forces from the blood flow, and the load concentrates on the fibrous cap and especially on its margin, the shoulder area. Rupture of plaque occurs when stress exceeds the critical level that the cap can withstand (Lee *et al.* 1997). Upon rupture, the highly thrombogenic lipid-rich core is exposed to circulating coagulative proteins leading to formation of a thrombus, and to clinical complications of atherosclerosis. However, if spontaneous fibrinolysis is active, the thrombus will remain non-occlusive and become part of the plaque. Even under such favorable conditions the repair process leads to plaque growth (Lee *et al.* 1997).

Interstitial collagen produced by SMCs confers most of the tensile strength on the fibrous cap (Lee *et al.* 1997), and is thus critical for the stability of a plaque. Typically, a stable plaque has a small lipid core and a thick fibrous cap, which is rich in SMCs and collagen, but poor in inflammatory cells. In contrast, a lesion with a large lipid core and a thin fibrous cap, containing only few SMCs and little collagen, but many inflammatory cells, is prone to rupture (Richardson *et al.* 1989). Inflammation is considered to occupy a central position in the rupture of atherosclerotic plaques (Libby 2002). The number of inflammatory cells, such as macrophages, T-lymphocytes, and mast cells, is increased at the sites of plaque rupture, and they destabilize the plaque by different mechanisms (Moreno *et al.* 1994; van der Wal *et al.* 1994; Kovanen *et al.* 1995). For instance, inflammatory cells are the most important source of matrix metalloproteinases (MMPs), that play a prominent role in the degradation of the ECM (Galis *et al.* 1994; Lijnen

2002). Moreover, cytokines, such as interferon-γ, released from T-lymphocytes can inhibit the production of collagen by SMC (Amento *et al.* 1991), and can also induce their apoptosis (Geng *et al.* 1998; Niemann-Jonsson *et al.* 2001).

Plaque erosion

Superficial plaque erosion without rupture is another frequent cause of coronary thrombosis (Farb *et al.* 1996; Arbustini *et al.* 1999). The exact mechanisms leading to plaque erosion have remained enigmatic, but ECs are thought to have a critical role in it. The EC monolayer forms a barrier between circulating blood and the vascular wall, and plays an essential role in the physiology of the vessel. Thus, a healthy endothelium tends to prevent thrombosis, inflammation, and spasm of the artery (Anderson 2003). Factors disturbing the integrity of endothelium, such as apoptosis of ECs, increase the risk of plaque erosion, exposing the highly thrombogenic ECM and causing thrombosis (Dimmeler *et al.* 1998; Dimmeler *et al.* 2002). Many of the classical risk factors of atherosclerosis, such as smoking (Wang *et al.* 2001), oxidized lipids (Dimmeler *et al.* 1997), and hyperglycemia (Ho *et al.* 2000) have been shown to be associated with EC apoptosis.

Mast cells

Mast cells were first characterized in the late 19th century by Paul Ehrlich, who observed cells with metachromically staining granules in connective tissue stained with an aniline dye. Ehrlich believed that the granules resulted from overfeeding of cells, and named the cells after the German word *Mästung*, to masticate or to feed (Ehrlich 1879). His ideas regarding granule origin soon proved wrong, but the somewhat misleading name remained. Ehrlich also described the localization of mast cells in association with blood vessels, inflamed tissues, nerves, and neoplastic tissues, and provided the first description of their degranulation. Mast cells have been shown to participate in various physiological and pathological processes, notably in allergic reactions (Williams *et al.* 2000), and also in the defense against parasites and bacteria (Wedemeyer *et al.* 2000), in gastric acid secretion (Metcalfe *et al.* 1981), in lipoprotein metabolism (Kovanen 1995), and in autoimmune diseases (Benoist *et al.* 2002).

Origin of mast cells and their differentiation

Mast cells are derived from multipotential hematopoietic stem cells originating in bone marrow (Rodewald *et al.* 1996; Li *et al.* 1999). Thus, undifferentiated mast cell progenitor cells leave the bone marrow and circulate in blood and lymphatic system before migrating to target tissues, notably to various mucosal surfaces and to skin, as well as to the arterial wall, where they proliferate and differentiate into granulated, mature mast cells (Wasserman 1990). Their migration and differentiation is influenced by several cytokines, such as interleukins -3, -4, -9, and the nerve growth factor (Madden *et al.* 1991; Mekori *et al.* 2000). However, the most important factor appears to be the stem cell factor (SCF), which is secreted locally by stromal cells, such as fibroblasts (Galli *et al.* 1993). The critical role of SCF is supported by the finding that SCF-deficient ($S1/S1^d$) and SCF-receptor c-kit deficient (W/W^v) mutant mice are largely devoid of mature mast cells (Kitamura *et al.* 1978; Kitamura *et al.* 1979). Moreover, SCF is the only cytokine that promotes the development of

mast cells in adult human system (Valent *et al.* 1992; Li *et al.* 1999).

Distribution and heterogeneity of mast cells

Almost all major organs and tissues of the body harbor mast cells. Most prevalent they are at the interface of the host and environment, reflecting their role in primary defense as the "sentinel cells". Thus, mast cells are often found in the proximity of blood and lymph vessels, in the skin, and in the mucosa of the upper and lower respiratory tracts and the gastrointestinal tract (Irani *et al.* 1986; Kitamura 1989; Galli 1990).

Mast cell populations are heterogeneous. Two major phenotypes, mucosal and connective tissue phenotype, have been identified in rodent tissues (Irani *et al.* 1986). Mucosal phenotype is predominant in the small bowel mucosa and connective tissue phenotype in the skin and on the serosal surfaces. These phenotypes differ in their morphological, histochemical and functional properties. For instance, heparin proteoglycans (PGs) appear only in connective tissue mast cells, whereas chondroitin sulphate PGs are found in both types of mast cells. The composition of neutral proteases in the secretory granules is also different, rat connective tissue mast cells contain chymase I and carboxypeptidase A, whereas rat mucosal mast cells contain tryptase-like chymase II (Kitamura 1989). The local microenvironment is able to regulate the mast cell phenotype (Austen *et al.* 2001). For instance, mouse mucosal-type mast cells, when cultured on fibroblasts adopt certain phenotypic characteristics of connective tissue type cells (Galli 1990).

Also human mast cells are heterogeneous. Two phenotypes have been identified based on the protease content: mast cells containing both tryptase and chymase belong to TC-type (MC_{TC}), whereas mast cells that contain only tryptase as their neutral protease belong to T-type (MC_{T}) (Wasserman 1990). MC_{TC} are the predominant type around blood vessels (Irani *et al.* 1986), and they are analogous with rodent connective tissue type mast cells.

Mast cell stimulation and degranulation

The most prominent funtional feature of mast cells is their ability, upon activation, to exocytose preformed mediators, such as the neutral proteases chymase and tryptase, heparin PGs and histamine, in their cytoplasmic secretory granules. Mast cell activation can be triggered by IgE-mediated crosslinking of IgE receptors (Fc-ε-RI) (Ishizaka *et al.* 1984), by histamine releasing factors secreted by neighboring T lymphocytes (Sedgwick *et al.* 1981) or macrophages (Liu *et al.* 1986), or by components of the complement system (C3a, C5a) (Metcalfe *et al.* 1981). Activated mast cells expel their cytoplasmic secretory granules in a process of exocytosis called degranulation. The secretory granules, consisting of preformed mediators bound to a network of negatively charged heparin and chondroitin sulfate PGs, become swollen, and their individual membranes fuse to form tubular degranulation channels in which the granules lie in chains (Rohlich *et al.* 1971). The degranulation channels then open to the extracellular fluid, and the soluble components of the granules, such as histamine, diffuse away. In contrast, as shown in rat serosal mast cells, the heparin PGs and the mast cell-specific neutral proteases (e.g., chymase and carboxypeptidase A) remain tightly bound to each other, forming proteolytically active extracellular

”granule remnants” (Kovanen 1993). The exocytosed remnants are eventually ingested by adjacent phagocytes, such as macrophages and SMCs (Kokkonen *et al.* 1987; Wang *et al.* 1995). The activated and degranulated mast cells reconstitute their lost preformed mediators through rapid onset of *de novo* synthesis, so allowing formation of new secretory granules. Finally, the recovered mast cells are ready to participate in a new process of activation and degranulation (Dvorak *et al.* 1986).

Mediators released from mast cells

Histamine

Mast cells are the major source of histamine in the tissues (Metcalfe *et al.* 1997). Histamine is a potent vasoactive agent, constrictor of bronchial smooth muscle, and plays a central role in the immediate allergic reactions (Repka-Ramirez *et al.* 2002). Histamine can also have pro-inflammatory and immune-modulatory effects (Bachert 2002). Within the mast cells histamine is stored in the secretory granules bound to heparin PGs (Rohlich *et al.* 1971). When mast cells are activated, histamine is secreted together with granules into extracellular space, where it rapidly dissociates from the heparin PGs. Histamine is also secreted at low levels by resting mast cells.

Mast cell proteoglycans

PGs have a long protein core into which side chains of highly sulfated glycosaminoglycans (GAG) of varying lenghts are covalently attached through a specific linkage region (Järveläinen *et al.* 2002). Mast cells contain two types of PGs, heparin and chondroitin PGs, which differ in the nature and sulphation of the GAG side chains. The heparin PGs make up about 90% of the rat serosal mast cell PGs (Katz *et al.* 1986) and 65% of human lung mast cell PGs (Stevens *et al.* 1988). In contrast to other intimal cells, which actively synthesize and secrete vascular PGs important for proper matrix assembly and structural integrity of vascular wall (Järveläinen *et al.* 2002), the mast cell produced PGs form the network of the intracellular secretory granules, in which neutral proteases are embedded. Eventually, due to mast cell activation and degranulation, also the mast cell derived PGs are released into the extracellular space. Interestingly, heparin PGs appear to protect the embedded proteases from inactivation, allowing them to remain active even in the extracellular fluid, containing physiological inhibitors of mast cell proteases (Pejler *et al.* 1995; Lindstedt *et al.* 2001a).

Mast cell proteases

Neutral proteases, i.e., enzymes that are proteolytically active at physiological pH, are the main product of mast cell biosynthetic machinery and may account for 20-50% of the total mast cell protein (Schwartz *et al.* 1984). Three main proteases have been purified from human mast cells: tryptase, chymase and carboxypeptidase A (CPA). In addition, TC-type mast cells also contain a cathepsin G-like protease (Schechter *et al.* 1990).

Tryptase is a trypsin-like neutral serine endoprotease, which is enzymatically active only in a form of tetramer and requires heparin or chondroitin sulphate for stability (Schwartz *et al.* 1986; Lindstedt *et al.* 1998). Tryptase cleaves protein and peptide substrates on the carboxyl side of Arg and Lys residues. A distinguishing feature for tryptase is resistance to inhibition by most natural protein inhibitors of serine proteinases (Smith *et al.* 1984). Actually, according to current notion, there are no physiological

inhibitors to this protease. Rather, tryptase becomes inactive in the extracellular fluid when the tetramer dissociates into monomers.

Chymase is a 28 kDa neutral serine endoprotease with a chymotrypsin-like activity. It cleaves the peptide or ester bonds at the carboxyl terminus of aromatic (Phe, Tyr, Trp) or branched-chain aliphatic (Leu, Ile, Val) amino acids. Accordingly, chymase has been shown to proteolyze several types of molecules present in the arterial intima. Chymase-mediated proteolysis can lead to activation or inactivation of bioactive molecules, such as matrix metalloproteinases (MMPs) (Saarinen *et al.* 1994; Johnson *et al.* 1998), TGF-β (Taipale *et al.* 1995), angiotensin I (Kokkonen *et al.* 1997), certain apolipoproteins (Lindstedt *et al.* 1996), and components of the ECM, such as fibronectin (Vartio *et al.* 1981) and vitronectin (Banovac *et al.* 1992).

Mast cell carboxypeptidase A is a 34.5 kDa neutral zinc metallo-exopeptidase, which cleaves C-terminal residues with hydrophobic side chains (Phe, Tyr, Leu). CPA has a complementary specificity to chymase, indicating that they can act sequentially to degrade target proteins (Woodbury *et al.* 1981; Kokkonen *et al.* 1986).

Other bioactive mediators secreted by mast cells

Mast cells synthesize and secrete a variety of mediators, which are vasoactive, or regulate inflammation and cellular growth. These include prostaglandin D_2, leukotrien C_4, leukotrien B_4, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and interleukins -4, -5, -6, and –13 (Young *et al.* 1987; Ra *et al.* 1994). TNF-α is a key cytokine in allergic inflammation, and is also a potent inducer of apoptosis (Rath *et al.* 1999). TGF-β regulates proliferation, differentiation and death of various cell types (Moustakas *et al.* 2002) and, among other functions, it is thought to play an anti-inflammatory role in atherogenesis (Lutgens *et al.* 2002).

Mast cells and atherosclerosis

Mast cells are present in normal human blood vessels as well as in atherosclerotic lesions, where they form a part of the inflammatory cell infiltrate (Stary 1990; Kaartinen *et al.* 1994a). The numbers of mast cells increase as atherosclerotic lesions develop (Kaartinen *et al.* 1998), but the exact triggers that lead to increased infiltration of mast cells are currently unknown. One of the possible candidates is eotaxin, a chemotactic factor for eosinophils, macrophages and mast cells, which is present in atherosclerotic lesions (Haley *et al.* 2000).

Inflammation is currently thought to play a central role in the initiation and progression of atherosclerosis (Ross 1999; Libby 2002). Since the number of activated mast cells is increased in culprit lesions in patients with unstable coronary syndromes (Kaartinen *et al.* 1998), the possibility exists that mast cells could participate in the pathogenesis of atherosclerosis. Indeed, there is increasing evidence that mast cells play a role in inflammatory cell recruitment, foam cell formation and genesis of unstable plaques (Kovanen 1995; Kelley *et al.* 2000).

Recruitment of inflammatory cells

Adhesion of circulating monocytes to endothelium is one of the earliest steps in atherosclerosis (Li *et al.* 1993b). Their entry into the arterial intima depends on the interaction with the adhesion molecules on the surface of ECs. As mentioned above,

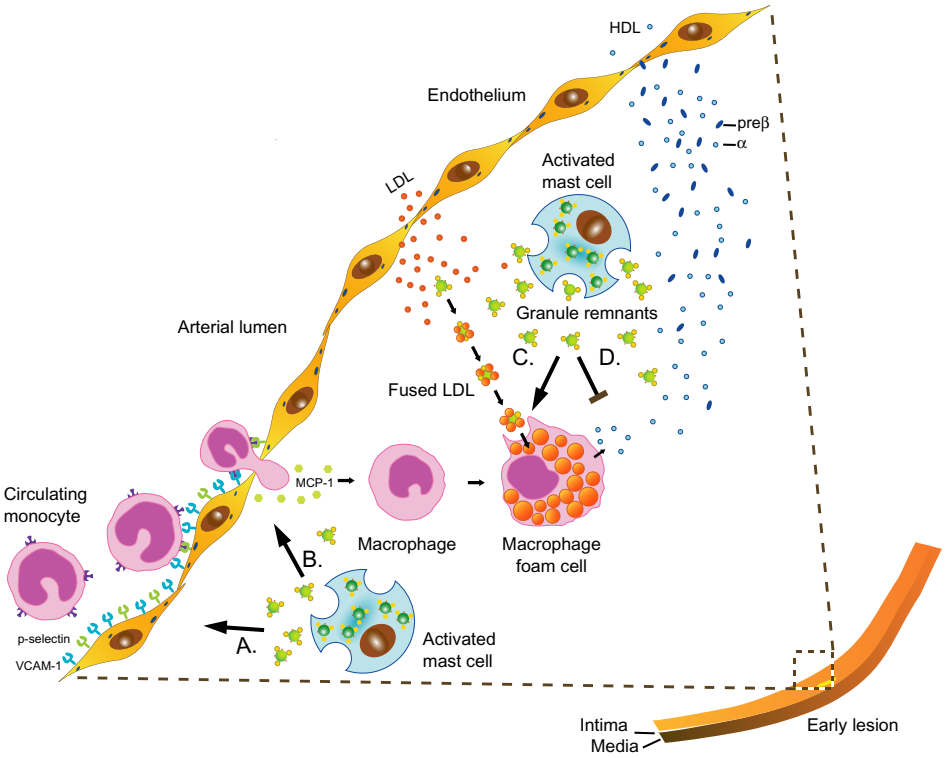

Figure 2. Effects of mast cells on the development of atherosclerotic lesions.

Mast cells have been proposed to play a role in recruitment of inflammatory cells to arterial intima by **A.)** increasing expression of adhesion molecules in endothelial cells and **B.)** increasing migration of monocytes into intima through stimulation of monocyte chemoattractant peptide-1 (MCP-1) production. Mast cells also increase cholesterol accumulation and foam cell formation in the intima by **C.)** increasing LDL influx into intima through granule remnant-mediated uptake of LDL and **D.)** decreasing cholesterol efflux from intima through degradation of preβ-HDL.

activated mast cells secrete a variety of pro-inflammatory substances (Bradding 1996), many of which, like TNF-α (Pober *et al.* 1986), tryptase (Compton *et al.* 2000), and histamine (Burns *et al.* 1999), can activate ECs to induce expression of adhesion molecules, such as P-selectin and VCAM-1, responsible for the recruitment of monocytes and lymphocytes (see Fig 2 A). Mast cells also stimulate MCP-1 production in fibroblasts through TNF-α and TGF-β (Gordon 2000), which, in turn, could increase monocyte penetration into intima. Thus, mast cells could participate in the initiation of atherosclerosis by recruiting monocytes and lymphocytes into the vascular intima (see Fig 2 B).

Neutrophil infiltration has recently been shown to occur in culprit lesions in acute coronary syndromes (Naruko *et al.* 2002), but the triggers of this phenomenon are unknown. Both human mast cell tryptase (He *et al.* 1997) and chymase (He *et al.* 1998) have been shown to lead to enhanced recruitment of neutrophils into the skin of guinea pigs, but the relevance of these findings in humans is unknown. The possibility exists that mast cells could play a role in neutrophil recruitment.

Mast cells contribute to foam cell formation

In atherosclerotic lesions, mast cells often reside in close association with macrophages and extracellular lipids, as well as at the sites of foam cell formation (Kaartinen *et al.* 1994b; Jeziorska *et al.* 1997), suggesting a role for mast cells in foam cell formation. The "balance theory" of atherogenesis suggests, that cholesterol, carried into the arterial intima by plasma LDL particles, is recirculated back to the circulation by plasma HDL particles. Thus, cholesterol accumulation and foam cell formation could result from an imbalance between these two processes (Kovanen 1990). Increasing evidence shows that mast cells can contribute to the transformation of macrophages and SMCs to foam cells *in vitro* by disturbing the balance between cholesterol uptake and efflux.

In order to enter the intima, LDL particles must cross the arterial endothelium, which acts as a barrier against lipoprotein particles (Stender *et al.* 1981). Histamine from mast cells is known to enhance vascular permeability to macromolecules (Wu *et al.* 1992), suggesting that activated mast cells could loosen the endothelial barrier and thus, increase the intimal concentration of LDL particles. So far, there is no experimental evidence supporting this notion in human coronary arteries. However, in an animal model of passive cutaneous anaphylaxis, a local activation of skin mast cells have been shown to result in acute and massive accumulation of blood LDL in the area in which the mast cells were activated to secrete vasoactive components, such as histamine (Ma *et al.* 1997).

Mast cells also increase the uptake of LDL by macrophages and SMCs. Briefly, the heparin PGs of mast cell granule remnants bind LDL-particles, which facilitates chymase-mediated degradation of apolipoprotein B in LDL. This results in the fusion of the LDL particles into larger lipid droplets and accumulation of LDL on granule remnants. Granule remnants coated with fused LDL particles are then phagocytosed by the macrophages and SMCs, leading to increased formation of foam cells (Kokkonen *et al.* 1987; Kokkonen *et al.* 1989; Wang *et al.* 1995; Piha *et al.* 1995). Moreover, soluble heparin PGs released from activated mast cells stimulate scavenger receptor- mediated uptake of LDL into macrophages (Lindstedt *et al.* 1992) (see Fig 2 C).

Efflux of cellular cholesterol is promoted by extracellular cholesterol acceptors, most notably small discoidal lipid-poor preβ-migrating high density lipoproteins (preβ-HDL) (Lee *et al.* 1992). Mast cell chymase can proteolyze the apolipoprotein A-I of preβ-HDL. This leads to reduced efflux of cholesterol from the foam cells, thus increasing cholesterol deposition in the macrophages present in atheromas (See Fig 2 D) (Lee *et al.* 1992; Lindstedt *et al.* 1996; Lee *et al.* 1999). Moreover, mast cell tryptase also degrades apoliproteins of HDL and blocks its function as an acceptor of cholesterol (Lee *et al.* 2002). The clinical significance of these mast cell -mediated effects on cholesterol metabolism is still unknown.

Mast cells and plaque destabilization

The most important mechanism of sudden onset of coronary syndromes, including unstable angina, acute myocardial infarction and sudden cardiac death, is erosion or rupture of a coronary atheroma (Fuster *et al.* 1992; Falk 1992; Virmani *et al.* 2000). Increased number of activated

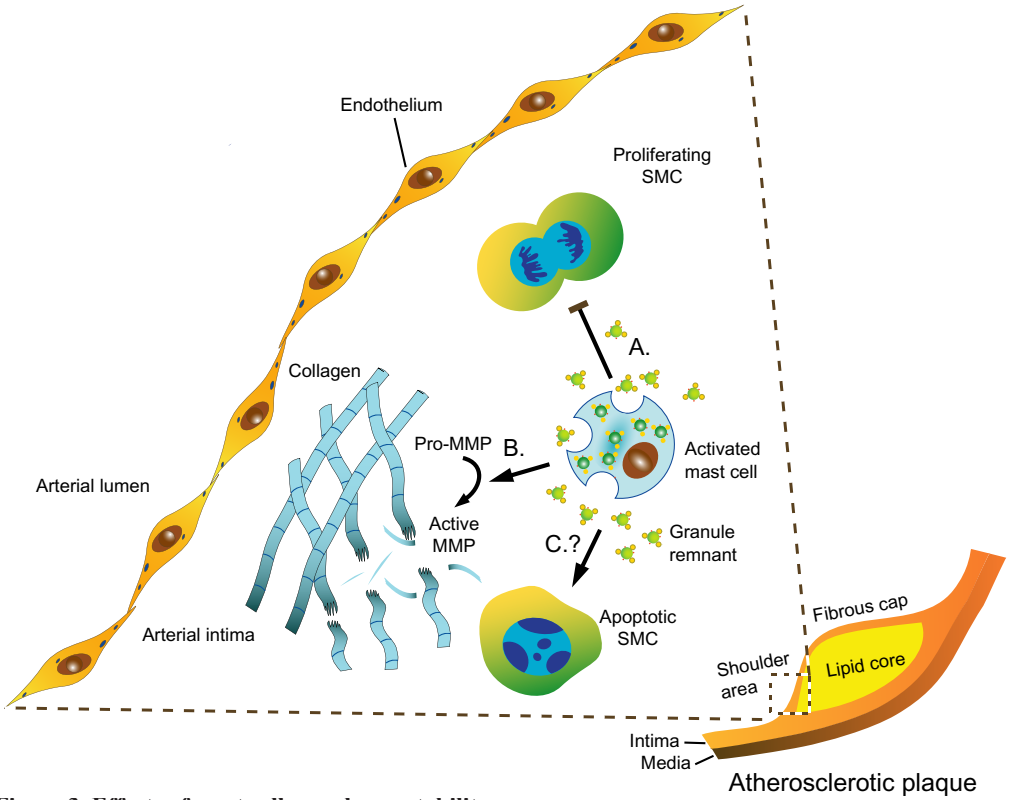

Figure 3. Effects of mast cells on plaque stability

Mast cells may reduce the stability of atherosclerotic plaques through several mechanisms. In vitro they have been shown to **A.)** inhibit proliferation of SMCs, and to **B.)** synthesize and activate MMPs capable of ECM degradation. Moreover, several mast cell-derived mediators, such as chymase and TNF-α are proapoptotic, raising the possibility that **C.)** mast cells might induce SMC apoptosis.

mast cells have been found at the site of the atheromatous rupture in patients who have died of acute myocardial infarction (Kovanen *et al.* 1995), and there is increasing evidence that mast cells could play a role in the destabilization of plaques.

The stability of the plaques depends on the thickness of the fibrous cap overlaying the lipid-rich core. The cap consists of SMCs and ECM, mostly collagen, produced and maintained by SMCs (Lee *et al.* 1997). Thus, the processes that decrease SMC number, inhibit SMC collagen synthesis or increase the ECM degradation tend to destabilize the plaque. A decrease in SMC number could be due to lowered proliferation of SMCs, or their increased elimination, e.g. by apoptosis.

Mast cell- derived heparin PGs have been shown to inhibit the proliferation of rat SMCs *in vitro* (Wang *et al.* 1999), suggesting that mast cells could participate in the regulation of SMC growth also in atherosclerosis (see Fig 3 A). However, since the rate of proliferation of SMCs in atherosclerotic lesions is rather low (Pickering *et al.* 1993), the clinical significance of such a mechanism in plaque stability is likely to be small. Under conditions of low proliferation, SMC numbers are largely controlled by cell death, either through necrosis or apoptosis. Some of the mediators released by mast cells are

proapoptotic, such as chymase, which induces cardiomyocyte apoptosis (Hara *et al.* 1999), and TNF-α, which triggers EC apoptosis (Slowik *et al.* 1997). This raises the possibility that mast cells could also induce apoptosis of SMCs and, by this way affect plaque stability (see Fig 3 C).

Matrix metalloproteinases (MMPs) are thought to play a prominent role in the degradation of the ECM components in atherosclerosis and to contribute to cap rupture and erosion (Galis *et al.* 1994; Lijnen 2002). By releasing TNF-α, a potent proinflammatory cytokine (Kaartinen *et al.* 1996), mast cells can induce synthesis and release of the 92 kDa gelatinase (MMP-9) from adjacent macrophages in paracrine fashion (Saren *et al.* 1996), and from the TNF-α containing mast cells in autocrine fashion (Baram *et al.* 2001). Moreover, TNF-α has also been shown to increase the expression of collagenase-1, stromelysin-1 and 92 kDa gelatinase in endothelial cells (Nelimarkka *et al.* 1998). Mast cells also synthesize and release interstitial collagenase (MMP-1) (Di Girolamo *et al.* 2000), which has been found in atherosclerotic lesions (Nikkari *et al.* 1995).

MMPs are synthesized and secreted as zymogens, i.e., as inactive proenzymes (proMMPs), and, consequently, have to be activated after secretion (Birkedal-Hansen *et al.* 1993). Chymase and tryptase, the two major neutral proteases of human mast cells, are both capable of activating MMPs *in vitro*, chymase activating proMMP-1 (Saarinen *et al.* 1994) and tryptase activating prostromelysin (proMMP-3) (Gruber *et al.* 1989). MMP-3, in addition to being a powerful matrix-degrading enzyme, can activate other proMMPs, thus triggering a more extensive degradation of the surrounding ECM (see Fig 3 B). In addition, chymase and tryptase can directly degrade ECM components, such as fibronectin and vitronectin (Vartio *et al.* 1981; Lohi *et al.* 1992).

Beneficial effects of mast cells in atherosclerosis

Mast cells may also have beneficial effects in atherosclerosis. Heparin PGs released from activated mast cells strongly prevent collagen-induced platelet aggregation (Lassila *et al.* 1997; Kauhanen *et al.* 2000), and may thus attenuate the thrombogenicity of the exposed matrix collagen. Mast cell tryptase can interfere with coagulation by degrading fibrinogen (Schwartz *et al.* 1985) and procoagulative kininogen (Maier *et al.* 1983), which could slow thrombus formation at the sites of plaque rupture. Moreover, rat serosal mast cells have been shown to block copper-mediated oxidation of LDL in vitro (Lindstedt *et al.* 1993). Thus, mast cells are also antithrombotic and antioxidative cells.

Apoptosis

Physiological role of apoptosis

Multicellular organisms need to efficiently dispose cells that are no longer needed or that have been damaged. This is accomplished through apoptosis, an active form of cell death first recognized in 1972 by Kerr, Wyllie and Currie in a landmark paper (Kerr *et al.* 1972). Apoptosis counterbalances cell division and cell migration in the maintenance of homeostasis of tissue mass, and it also contributes to many fundamental biological processes, including morphogenesis (Koseki *et al.* 1992; Raff *et al.* 1993) and remodeling of mature tissues (Polunovsky *et al.* 1993). Apoptosis is also needed to purge the body from pathogen-invaded cells and to

eliminate auto-aggressive immune cells. Inadequate or excessive apoptosis may lead to pathological processes, including developmental defects, autoimmune diseases, neurodegenerative diseases, or cancer. Lately, apoptosis has also generated interest in the pathophysiology of atherosclerosis (Kockx *et al.* 1998; Mallat *et al.* 2001; Bennett 2002).

Apoptosis is distinct from the conventional type of cell death, i.e., necrosis, in that it has different morphological and biochemical properties. Necrosis is a violent form of cell death and can be caused by a range of noxious chemicals, biological agents, or physical damage. Necrosis is characterized by swelling of cellular organelles and cytoplasm, rupture of the cellular membranes and disintegration of the cell. During necrosis the cytosolic proteins of the dying cell are released into the intercellular space, initiating an inflammatory response (Wyllie 1997). In contrast to necrosis, apoptosis is a coordinated process requiring active participation of the dying cell. The classical morphological signs of apoptosis include cell shrinkage, membrane blebbing, chromatin condensation, and fragmentation of the nucleus into discrete masses scattered throughout the cell cytoplasm (Wyllie *et al.* 1980). During apoptosis the phospholipid content of the outer cellular membrane changes and phosphatidylserine molecules become exposed (Fadok *et al.* 1992). Surrounding cells and macrophages recognize this change and rapidly engulf the apoptotic cell or its membrane-enclosed remains, the apoptotic bodies. As a consequence, apoptosis evokes little if any inflammatory response, unless the clearance of apoptotic bodies fails, in which case secondary necrosis ensues and inflammation is initiated. Secondary necrosis together with the observation

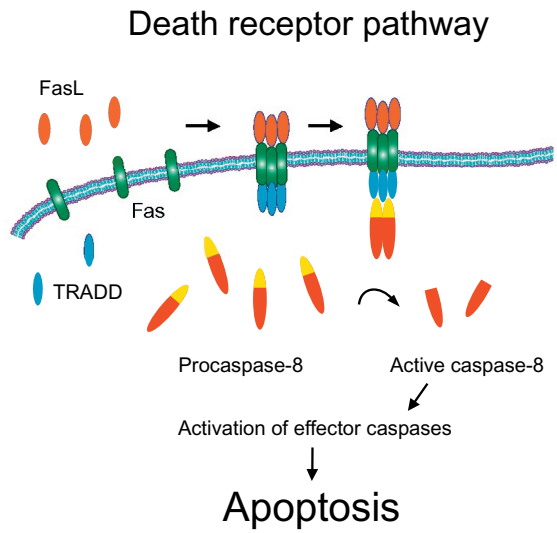

Figure 4. Death receptor-mediated apoptotic pathway
Activation of the plasma membrane death receptors, such as Fas or TNFαR, by their ligands leads to adapter protein (e.g. TRADD) mediated recruitment and subsequent activation of initiator caspase-8. Active caspase-8 then activates downstream effector caspases, which are responsible for the proteolytic cleavage of cytoskeletal components, nuclear proteins and lamins, leading to changes characteristic for apoptosis and finally to cell disassembly .

that same stimuli can induce both apoptosis and necrosis at different conditions or even simultaneously (Dypbukt *et al.* 1994), makes the boundary between apoptosis and necrosis somewhat blurred, but in most cases a distinction is possible.

Regulation of apoptosis

Apoptosis is tightly regulated to avoid inadvertent self-destruction of the cells (Hengartner 2000). Apoptosis can be initiated by a wide variety of signals, including activation of death receptors, γ-irradiation, hypoxia and UV light. These proapoptotic signals are processed by the cell and evaluated against anti-apoptotic factors, such as growth factor stimulation, hormones and attachment to neighboring cells before the decision to commit apoptosis is made. Most cells need constant

anti-apoptotic stimulation to survive, and withdrawal of these signals leads to apoptosis. This enables multicellular organisms to dispose cells that either have lost their anchorage or are located in a nonpermissive environment.

There are two major pathways leading to the initiation of apoptosis: the death receptor or extrinsic pathway, and the mitochondrial or intrinsic pathway. Activation of the plasma membrane death receptors of the tumor necrosis factor receptor (TNF-R) family, such as Fas or TNFαR, leads to adapter protein -mediated recruitment and subsequent activation of the initiator caspase-8 (Chen *et al.* 2002). Caspases form a family of highly conserved cysteine proteases that initiate and execute the program of cell death (Cohen 1997). Activated caspase-8 activates downstream effector caspases (caspase-3, -6, -7) that are directly responsible for the proteolytic cleavage of cytoskeletal components, nuclear proteins and lamins, which then leads to changes characteristic for apoptosis and finally to cell disassembly (see Fig 4). In addition to death receptors, caspase-8 can also be activated by unligated integrins (Stupack *et al.* 2001). Thus, by overexpressing cytoplasmic domains of β1 and β3 integrins the authors were able to induce caspase-8 mediated apoptosis of COS7 cells.

In the mitochondrial pathway, apoptosis is initiated by a release of mitochondrial cytochrome c and other pro-apoptotic molecules into the cytoplasm (Chen *et al.* 2002; Mayer *et al.* 2003). The association of cytochrome c with an adaptor molecule Apaf-1 and procaspase-9 leads to the formation of active caspase-9 and subsequent effector caspase activation (see Fig 5). The release of cytochrome c from mitochondria is regulated by the balance

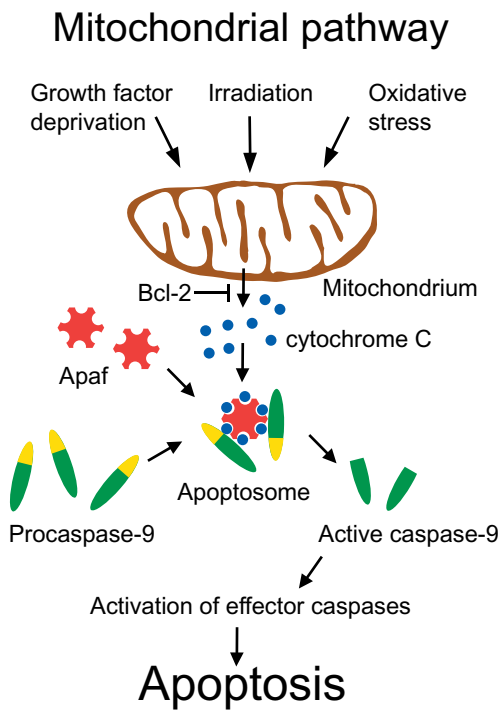

Figure 5. Mitochondrial apoptotic pathway
Diverse apoptotic signals, such as DNA damage, irradiation, growth factor deprivation, and loss of adhesion to neighboring cells are able to initiate apoptosis by changing the balance between the pro- and antiapoptotic members of bcl-2 superfamily. Shift in balance to proapoptotic members leads to release of mitochondrial cytochrome C and other pro-apoptotic molecules into the cytoplasm. Together with apaf-1 cytochrome C binds procaspase-9 forming an apoptosome, which is able to activate caspase-9, resulting in effector caspase activation.

of the pro- and anti-apoptotic proteins of the bcl-2 superfamily. Signals as diverse as DNA damage, irradiation, growth factor deprivation, and loss of adhesion to neighboring cells affect the balance within bcl-2 family, and can lead to apoptosis. Although the death receptor and the mitochondrial pathway are distinct, there is crosstalk between them. For instance, activated caspase-8 can activate pro-apoptotic bid of the bcl-2 family, leading to activation of the caspase-9 pathway (Li *et al.* 1998).

Apoptosis in the vessel wall

Apoptosis is important in the regulation of homeostasis and remodeling, as well as in a variety of diseases. Apoptosis of SMCs is a prominent feature of blood vessel remodeling during normal development (Walsh *et al.* 2000), underlying for instance closure of ductus arteriosus (Slomp *et al.* 1997), reduction of the lumen size of umbilical arteries after the birth (Cho *et al.* 1995), and remodeling of the embryonic mammalian pharyngeal arch artery system (Molin *et al.* 2002). Apoptosis occurs also at the branch points of great arteries arising from the aortic arch in human neonates, when they are exposed to disturbed blood flow (Kim *et al.* 2000). Apoptosis during remodeling appears to be triggered by changes in blood flow and wall tension. Both decreased flow, perturbations in the flow (Cho *et al.* 1997), as well as changes in the wall tension (Bayer *et al.* 1999) induce apoptosis of SMCs, allowing the vasculature to adapt to new conditions. In contrast, normal vessel wall demonstrates very low apoptotic and mitotic indices.

Extensive research on balloon angioplasty has provided a wealth of information regarding arterial injury and apoptosis. In animal models, a burst of medial SMC apoptosis is observed early after balloon distention injury (Perlman *et al.* 1997), resulting in a decreased cellularity. Days and weeks later this initial apoptosis is followed by a second wave of SMC apoptosis in the developing neointima (Han *et al.* 1995). Apoptosis induced by arterial injury appears to be related to the extent of the insult, as increased balloon-to-artery ratios result in greater frequencies of SMC apoptosis (Rivard *et al.* 1999).

Apoptosis of vascular SMC is also thought to participate in development of aneurysms. Formation of arterial aneurysms involves degradation of collagen, elastin, and vascular PGs, the major structural elements of the vessel wall, and loss of SMCs from the vessel media. MMPs and other matrix degrading enzymes are thought to play a critical role in aneurysm formation. Lately apoptosis of SMCs has emerged as a possible additional factor (Thompson *et al.* 1997). Thus, apoptosis of SMCs has been shown to occur in abdominal (Holmes *et al.* 1996) and cerebral (Kondo *et al.* 1998) aneurysms and aneurysm formation is associated with an increase in pro-apoptotic molecules (Henderson *et al.* 1999). This would weaken the wall and favor aneurysm formation due to loss of synthesis of ECM components, such as collagen, elastin and PGs.

Apoptosis in atherosclerotic lesions

Apoptosis has recently generated much interest among scientists studying the pathogenesis of atherosclerosis (Kockx *et al.* 2000; Mallat *et al.* 2001; Bennett 2002). Thus, apoptosis has been proposed to play a role in the development of atherosclerotic plaques (Bennett *et al.* 1995; Kockx *et al.* 1998), in the generation of procoagulant microparticles (Mallat *et al.* 1999), and in plaque rupture (Kolodgie *et al.* 2001). Apoptosis of SMCs, macrophages and ECs has been observed in atherosclerotic lesions (Kockx *et al.* 1998; Tricot *et al.* 2000), and these apoptotic cells are often associated with the infiltrates of inflammatory cells (Crisby *et al.* 1997). The frequency of apoptotic cell death seems to increase as atherosclerotic plaques develop. Early lesions tend to have few apoptotic cells, whereas advanced atherosclerotic plaques show increased apoptosis (Kockx *et al.* 1998). Moreover, increased SMC apopto-

sis has been detected in unstable lesions compared with stable lesions of human coronary arteries (Bauriedel *et al.* 1999).

Apoptosis of smooth muscle cells

The SMCs produce most of the ECM in plaques. Thus, their apoptosis would be detrimental to plaque stability, as it would lead to the loss of collagen and, subsequently, to the loss of the mechanical integrity of the plaque. SMCs from human atherosclerotic plaques show relatively low values of cell replication (Pickering *et al.* 1993), and thus modest increase in the apoptotic rate could lead to a significant decrease in SMC content. An important observation is that the SMCs isolated from human coronary atherosclerotic plaques are more prone to apoptosis compared to SMCs from normal vessels (Bennett *et al.* 1995), and that they show strong expression of the pro-apoptotic protein bax (Kockx *et al.* 1998).

Several possible triggers of SMC apoptosis are present in atherosclerotic lesions. These include inflammatory cells, certain cytokines, and oxidized lipids (Geng *et al.* 2002). Inflammatory cells can trigger SMC apoptosis *in vitro* by cell-cell interactions (Geng *et al.* 1997; Boyle *et al.* 2002; Seshiah *et al.* 2002), and also by secreting pro-apoptotic cytokines, such as TNF-α (Niemann-Jonsson *et al.* 2001). Modified lipids, especially oxidized LDL, have been shown to induce SMC apoptosis *in vitro* (Taguchi *et al.* 2000). The importance of these mechanisms in triggering SMC apoptosis in atherosclerotic lesions *in vivo* is still unknown. However, the close spatial relationship between apoptotic SMCs and inflammatory cells (Kockx *et al.* 1998) suggests, that inflammatory mechanisms are likely to participate in the regulation of SMC apoptosis *in vivo*.

Apoptosis of macrophages

Macrophages promote apoptosis of SMCs and are responsible for metalloproteinase-mediated collagen breakdown in atherosclerotic lesions. Accordingly, apoptosis of macrophages in atherosclerotic lesions is expected to have a positive effect on plaque stability (Kockx *et al.* 2000). However, macrophages are important phagocytotic cells and their loss would also decrease the scavenging of the apoptotic bodies in plaques, leading to extracellular accumulation of the apoptotic bodies with ensuing complement and thrombin activation. Moreover, excessive apoptosis of macrophage foam cells could contribute to the formation of the lipid core (Hegyi *et al.* 1996).

Macrophages are resistant to several triggers of apoptosis, including FasL (Perlman *et al.* 1999), and they tolerate high levels of oxidative stress. The exact triggers of macrophage apoptosis in atherosclerotic lesions *in vivo* are not known, and it is likely that several factors, such as oxidative stress and cytokines, are acting together to induce macrophage apoptosis. Tumor suppressor gene p53 mediated apoptosis is supposed to be important in macrophage death. APOE*3-leiden mice, an animal model for human-like atherosclerosis, show decreased apoptosis and increased lesion area, when their macrophages are rendered deficient of p53 through irradiation and bone marrow reconstitution from p53(-/-) donor mice (van Vlijmen *et al.* 2001).

Apoptosis of endothelial cells

EC apoptosis has been suggested to contribute to atherogenesis, plaque erosion and acute coronary syndromes (Dimmeler *et al.* 2002). Atherosclerotic plaques tend to develop in the areas with increased EC turnover rates, and apoptosis has been

suggested to be involved in this (Dimmeler *et al.* 1998; Choy *et al.* 2001). EC apoptosis is also one of the possible mechanisms leading to plaque erosion (Dimmeler *et al.* 2002). Apoptotic ECs are procoagulant (Bombeli *et al.* 1997) and may thus contribute to thrombosis in acute coronary events (Dimmeler *et al.* 2002).

ECs in the normal arterial wall are very resistant to apoptosis (Polunovsky *et al.* 1994; Bach *et al.* 1997), suggesting that special pathological conditions are required for the induction of EC apoptosis. Shear stress appears to be an important anti-apoptotic factor for ECs (Dimmeler *et al.* 1996), and in human atherosclerotic lesions, the incidence of EC apoptosis is higher in the downstream parts of plaques, where low flow and low shear stress prevail (Tricot *et al.* 2000). Interestingly, most of the classic risk factors for atherosclerosis, such as oxidized LDL (Dimmeler *et al.* 1997), smoking (Wang *et al.* 2001), and high glucose concentration (Ho *et al.* 2000) have been shown to be associated with EC apoptosis *in vitro.* However, the proof of the clinical relevance of EC apoptosis in atherosclerosis is still missing (Dimmeler *et al.* 2002).

AIMS OF THE STUDY

Mast cells form a fraction of the inflammatory cell infiltrate in atherosclerotic lesions, and an increased number of activated mast cells is present at the sites of plaque rupture or erosion in patients that have died of acute myocardial infarction (Kovanen *et al.* 1995). These observations raise the possibility that mast cells might affect the stability of coronary atheromas.

One of the possible mechanisms causing the rupture or erosion of coronary plaques is the apoptotic loss of SMCs and ECs, respectively. In atherosclerotic plaques, apoptotic SMCs are often located in the areas rich in mast cells and other inflammatory cells (Crisby *et al.* 1997; Kockx *et al.* 1998), raising the possibility that mast cells contribute to apoptosis of SMCs. Moreover, several mast cell-derived mediators, such as chymase (Hara *et al.* 1999) and TNF-α (Slowik *et al.* 1997) have proapoptotic properties.

The aim of this thesis work was to study the possible role of mast cells in inducing SMC and EC apoptosis. Specifically, we wanted to answer the following questions:

1. Are mast cells able to induce apoptosis of cultured SMCs and/or ECs?
2. If so, what are the mast cell-derived pro-apoptotic factors and by which mechanisms is apoptosis induced.
3. Are there other mechanisms by which mast cells can affect the growth and function of cultured SMCs?

METHODS

Isolation of mast cell-derived factors

Animals

Male Wistar rats (300 – 500g) were from the Laboratory Animal Center of the University of Helsinki. The rats were treated in accordance with institutional guidelines, which had been approved by the institutional Ethics Committee.

Isolation and stimulation of mast cells

Serosal mast cells were isolated from pleural and peritoneal cavities of rats as described previously (Kokkonen *et al.* 1985), and stimulated with compound 48/80, a noncytotoxic mast cell-specific stimulator (Lindstedt *et al.* 1992). After stimulation, the cells were sedimented by centrifugation at 800g for 5 minutes. The supernatant, which contains all the material released from the stimulated mast cells, will be referred to in the text as "mast cell releasate" (see Fig 6). The degree of mast cell degranulation was determined by measuring the histamine content of the releasate (Kokkonen *et al.* 1985).

Preparation of granule remnants and granule remnant-free releasate

To sediment granule remnants, the mast cell releasate was centrifuged at 13,000g for 10 minutes. After centrifugation, the supernatant, which contains all the soluble material of the mast cell releasate, was collected, and used as "granule remnant-free releasate". The sedimented granule remnants were washed twice with water, resuspended in PBS, and used as "granule remnants" (see Fig 6). The concentration of the granule remnants is expressed in terms of their total protein content, or of their chymase activity, using N-benzoyl-tyrosine ethyl ester (BTEE) as substrate (Woodbury *et al.* 1981).

Purification of heparin proteoglycans, chymase and carboxypeptidase A

To dissociate the two neutral proteases chymase and carboxypeptidase A (CPA) from the heparin PGs, isolated granule remnants were incubated in high-salt buffer consisting of 10 mmol/L phosphate buffer supplemented with 2 mol/L KCl, pH 7.0. The mixture was then applied to a Sephacryl S-200 HR column, and eluted with the same high salt buffer. Fractions containing Alcian blue-reactive material were collected, dialyzed extensively against water, concentrated with a Centricon 10 filter, and used in the experiments (Yurt *et al.* 1977). These heparin PG fractions were devoid of any protease activity, as determined by a sensitive method involving analysis of the proteolytic products of angiotensin I by reverse-phase high-performance liquid chromatography (RP-HPLC) (Kokkonen *et al.* 1997). Briefly, angiotensin I, a peptide highly susceptible to chymase degradation, was incubated with the sample for 30 min and the peptides were then isolated and analyzed on RP-HPLC. Angiotensin I-derived products were identified by comparison of their retention times with those of synthetic standards.

Fractions containing chymase or CPA activity were collected separately, and further purified on a HiTrap heparin-

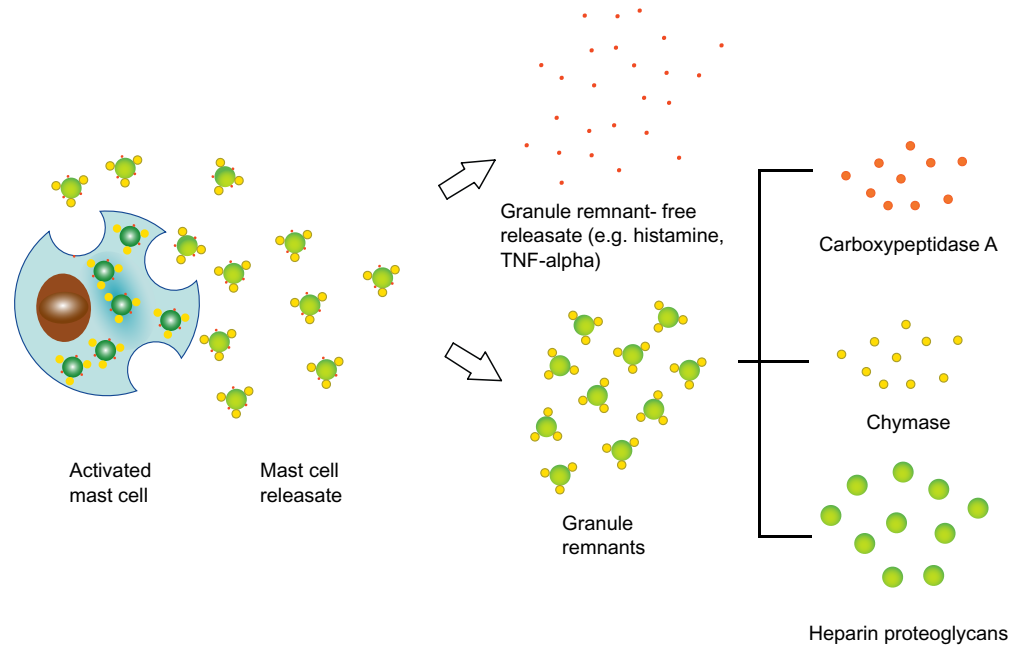

Figure 6. Rat serosal mast cell- derived mediators

Upon stimulation, mast cells exocytose preformed mediators in secretory granules (mast cell releasate). Histamine and other soluble mediators diffuse away, whereas heparin proteoglycans and the mast cell-specific neutral proteases remain tightly bound to each other, forming proteolytically active extracellular "granule remnants" Components of the granule remnants, e.g. chymase, CPA and heparin proteoglycans can be dissociated from each other by high salt and purified by gel filtration and affinity chromatography.

Sepharose column. The purity of chymase and of CPA was determined by SDS-PAGE and RP-HPLC (Kokkonen *et al.* 1997). The purified heparin PGs, chymase, and CPA were stored at -70°C until use.

Production of chymase-generated fibronectin degradation products

To produce FN degradation products, commercial FN was incubated at 37ºC either for 30 min with proteolytically active mast cell granule remnants containing 40 BTEE units/mL of chymase, or for 2.5 minutes with 20 BTEE units/mL of purified chymase. After incubation, chymase was inactivated by either removing the granule remnants by centrifugation (20 min, 15 000 g) or adding PMSF (1 mmol/L). The absence of contaminating active chymase in the FN degradation products was verified by a chromogenic substrate (BTEE) and RP-HPLC method (Kokkonen *et al.* 1997).

Cells

Isolation, culture and growth arrest of rat aortic smooth muscle cells

Aortic SMCs were isolated from male Wistar rats as previously described (Wang *et al.* 1999). The cells were seeded into 25 cm^2 Falcon polystyrene tissue culture flasks at a density of 2 x 10^4 cells/cm^2 in 5 mL of RPMI 1640 culture medium supplemented with 2 mmol/L L-glutamine, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 10% fetal calf serum (FCS), and, at confluence, were subcultured (1:2) up to nine times. SMCs of the

5th to 9th passages were used in the experiments. To obtain growth-arrested SMCs, sparsely seeded SMCs were cultured to a subconfluent cell density in the above culture medium containing 10% FCS, after which they were growth-arrested in 0.4% FCS-containing medium for 48 hours (Castellot, Jr. *et al.* 1989).

Culture of human coronary arterial smooth muscle cells

Human coronary artery SMCs were obtained from Clonetics, cultured in SmGM-3 medium and, at confluence, subcultured up to three times, as recommended by the manufacturer.

Isolation and culture of rat myocardial endothelial cells

ECs were isolated from rat myocardium and cultured as previously described (Piper *et al.* 1990). The cells were identified as ECs by their positive immunostaining for von Willebrand factor and uptake of DiI-labeled acetylated LDL, and the contamination with other cardiac cells was found to be less than 1%. The ECs were used in experiments between the 2nd and 4th passages.

Detection of apoptosis

Microscopic analysis of apoptosis

Cell smears were stained by the standard May-Grünwald-Giemsa method, after which apoptotic cells were identified by the following criteria: condensation of cytoplasm, cell surface blebbing, compaction of chromatin, and fragmentation of the nucleus into discrete masses scattered throughout the cell cytoplasm (Wyllie *et al.* 1980). Alternatively, cell smears were stained with 25 μg/mL propidium iodide, or with 1 mg/mL Hoechst 33342 after which the cells were viewed under fluorescence microscope, and cells with fragmented nuclei were considered as apoptotic.

Annexin-V-FLUOS staining

Translocation of phosphatidyl serine from the inner layer of plasma membrane to the outer layer is a common feature in many apoptotic cells (Fadok *et al.* 1998). Presence of phosphatidyl serine on the cell surface was detected by Annexin-V-FLUOS staining (0.1 μg/mL) according to the manufacturers recommendation.

TUNEL- assay

Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) with an ApoTag In Situ Apoptosis Detection Kit. Briefly, apoptotic cells were labeled with digoxigenin-conjugated dUTP and terminal deoxyribonucleotide transferase, the labeled DNA fragments were stained with anti-digoxigenin monoclonal antibody linked with fluorescein , and viewed under fluorescence microscope.

Flow cytometric analysis of apoptosis and cell cycle

The cellular DNA content was determined by flow cytometric analysis of PI-labeled cells (Leszczynski 1995). Briefly, the SMCs attached to the culture dishes were detached with trypsin and combined with the cells floating in the culture medium. The cells were then fixed with methanol, incubated with 100 units/mL of RNAase and, finally, stained with propidium iodide (PI). After staining, the cells were analyzed with a FACScan, using CellFit 2.02 software. Fluorescence was measured from 10 000 events in list mode. Gating on FL2-A versus FL2-W was used to remove doublets. Apoptotic nuclei were identified as a subgenomic DNA peak and were distinguished from cell debris by both their forward light scatter and the fluorescence

of PI. The cell cycle distribution was analysed by CellFit 2.02 software.

DNA fragmentation analysis

DNA was extracted by a standard method (Strauss 1987), and equal amounts of DNA from each sample were subjected to electrophoresis on 1.5% agarose gel for 3 hours at 80 V in TBE buffer. The gel was stained with ethidium bromide, and photographed under ultraviolet light.

Analysis of apoptotic mechanism

Caspase assays

Activity of caspases -3, -6, -8, and -9 were analyzed by using fluorometric assay kits. Briefly, the cells were lysed and the lysates were incubated with a caspase-specific peptide conjugated to the fluorescent reporter molecule. The cleavage of the peptide by the caspase releases the fluorochrome that, when excited with light, emits fluorescence directly proportional to the caspase enzymatic activity in the cell lysate. Fluorescence was detected by a microplate reader.

Reverse transcriptase -PCR analysis of bcl-2 and bax

Total RNA was extracted from EC and SMC monolayers, using an ultrapure TRIzol reagent; 1-4 μg of RNA was then reverse-transcribed into cDNA, using a Superscript ™ pre-amplification system. The cDNA obtained was further amplified by PCR with specific oligonucleotides (see ***Study IV, Table 1***) Competitive RT-PCR was performed as previously described (Lindstedt *et al.* 2001b). The competitor DNA for bcl-2 was obtained by inserting an 143 bp external DNA fragment into the BamHI site, whereas the competitor DNA for bax was obtained by inserting an 124 bp external DNA fragment into the NheI site. The use of equal amounts of mRNA in the RT-PCR assays was confirmed by analyzing the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR fragments obtained were verified to represent the corresponding targets either by specific restriction enzyme treatment or by DNA sequencing. The PCR products were quantitated with a Gel Doc 2000 gel documentation system.

Detection of cytoplasmic and mitochondrial cytochrome c

ECs were cultured in the presence or absence of mast cell granule remnants for 6 hours after which the cells were collected and disrupted with a Dounce homogenizer. Cytoplasm and mitochondrial fractions were isolated as previously described (Tafani *et al.* 2000) and were further subjected to SDS-PAGE and Western blotting, after which the level of cytochrome c in the subfractions was measured using a polyclonal antibody against cytochrome c. The amount of cytochrome c was quantified with a Gel Doc 2000 gel documentation system.

Immunoblotting

The cells were lysed in sodium orthovanadate buffer (150 mmol/L NaCl, 10 mmol/L Tris, pH 7.6, 1% Triton X-100, 5 mmol/L EDTA, 1 mmol/L PMSF, 2 mmol/L benzamidine, 2.5 μM aprotinin, 2.5 μM leupeptin, and 1 mmol/L sodium orthovanadate). For each sample, 40 μg protein was electrophoresed on 12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with either anti-FAK, anti-Akt, anti-p-FAK, anti-p-Akt, 4G10 anti-phosphotyrosine or anti-NFκB antibody. Immunoreactive bands were visualized through enchanced chemiluminescence with either horserad-

ish peroxidase (HRP) conjugated rabbit anti-mouse IgG (anti-FAK, anti-Akt and 4G10), HRP-conjugated goat anti-rabbit IgG (anti-p-FAK, anti-NFκB), or HRP-conjugated rabbit anti-sheep IgG (anti-p-Akt). The immunoblots were quantitated by densitometric scanning with a Gel Doc 2000 gel documentation system.

Immunostaining of cells

SMCs and ECs were cultured to subconfluent monolayers on Thermanox coverslips, exposed to granule remnants or purified chymase and fixed with 4% paraformaldehyde for 10 minutes at room temperature. FN was detected using a polyclonal antibody and a rabbit-anti-goat secondary antibody conjugated with peroxidase. P-FAK was detected with an anti-p-FAK antibody and visualized with an Alexa Fluor (546)-labeled goat anti rabbit antibody. NFκB was detected with anti-NFκB antibody and visualized with biotin-conjugated goat anti-rabbit antibody and FITC- conjugated streptavidin. bcl-2 was detected with anti-bcl-2 antibody, and bax with anti-bax antibody. Both were amplified with a biotinylated secondary antibody and visualized with HRP-conjugated streptavidin with 3-amino-9-ethyl carbazole (AEC) as substrate.

Immunostaining of smooth muscle cell-derived ECM

SMC-derived ECM was prepared by sequential extraction with sodium deoxycholate and hypotonic buffer in the presence of protease inhibitor as previously described (Hedman *et al.* 1979), except that the SMCs were cultured on Thermanox coverslips. FN was detected using a polyclonal antibody and a rabbit-anti-goat secondary antibody conjugated with peroxidase. TGF-β1 was then detected with a polyclonal antibody against TGF-β1 using biotin-streptavidin amplification and peroxidase staining, as recommended by the manufacturer.

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted as previously described (Andrews *et al.* 1991) and electrophoretic mobility shift assay was performed with minor modifications (Pentikäinen *et al.* 2002). Briefly, 10 μg nuclear extracts were incubated for 60 minutes at room temperature with 50 000 cpm of ^{32}P-labeled NFκB consensus oligonucleotide probe, which has a binding site for NFκB/c-Rel homodimeric and heterodimeric complexes. The samples were run on 4% polyacrylamide gels in 0.25 x TBE buffer, dried and exposed to light sensitive film at –70°C. Competition was performed by the addition of 100-fold excess nonradioactive oligonucleotide competitors before addition of radioactive probe. The sequence of NFκB oligonucleotides was as follows:

Consensus

5’- AGT TGA GGG GAC TTT CCC AGG C – 3’

Unlabeled mutant

5’- AGT TGA GGC GAC TTT CCC AGG C – 3’

Other assays

Protein was determined by the standard Lowry procedure (Lowry *et al.* 1951). The glycosaminoglycan content of heparin PGs was determined by assaying Alcian blue-reactive material with commercial heparin as standard (Bartold *et al.* 1985). The quantity of TGF-β1 was determined by ELISA, as recommended by the manufacturer.

Polyacrylamide gel electrophoresis and immunoblotting of TGF-β1

Gradient (4 to 20%) sodium deoxycholate-polyacrylamide gel electrophoresis for

detection of active TGF-β1 was carried out as described (Taipale *et al.* 1994). Active TGF-β1 was detected with a polyclonal antibody against TGF-β1, using biotin-streptavidin amplification and ECL detection (Taipale *et al.* 1992).

Competitive RT-PCR analysis of TGF-β1, PAI-1, TβRI, TβRII and collagen type I and III mRNA

Total RNA was extracted from subconfluent SMCs using an ultra pure TRIzol reagent; 1 μg of RNA was then reverse-transcribed into cDNA using a Superscript ™ pre-amplification system. The cDNA obtained was further amplified by PCR with specific oligonucleotides (see ***Study II, Methods***). The PCR fragments obtained were verified to represent the corresponding targets by specific restriction enzyme treatment and quantitated with a Gel Doc 2000 gel documentation system. For competitive RT-PCR, competitor DNAs were prepared by insertion of a 125-bp external DNA fragment at the *Pst* I site for TGF-β1, a 129-bp fragment at the *Stu* I site for collagen type I, and a 133-bp fragment at the *Bam*H I site for collagen type III, respectively. The PCR products for the target and its competitor were 350 bp and 475 bp for TGF-β1, 266 bp and 395 bp for collagen I, and 312 bp and 445 bp for collagen type III, respectively.

Determination of DNA synthesis

DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine (1 μCi/mL) into the trichloroacetic acid (TCA)- precipitable material of the SMCs (Wang *et al.* 1999). Cell viability of chymase-treated SMCs was monitored by phase-contrast microscopy with and without trypan blue staining of cells.

Statistical analysis

Data, shown as means ± SEM, were analyzed with Student's *t* test for determination of the significance of differences, which were considered to be statistically significant at a P value of less than 0.05.

RESULTS

Mast cells and smooth muscle cell apoptosis

Activated mast cells induce smooth muscle cell apoptosis

To investigate whether activated rat serosal mast cells are able to induce smooth muscle cell (SMC) apoptosis, we prepared mast cell releasate containing all the substances released by activated mast cells. Growth-arrested rat aortic SMCs were exposed to increasing concentrations of mast cell releasate for 24 hours and their DNA content was analysed by flow cytometry. Cells having hypodiploid DNA peaks were considered apoptotic. As the concentration of mast cell releasate increased, the percentage of apoptotic SMCs increased in a dose-dependent manner, reaching 11,4% with releasate derived from 3.6 x 10^5 mast cells. (***Study I, Figure 1 A***) The presence of apoptotic SMCs in mast cell releasate-treated samples was further confirmed by appearance of cells with fragmented nuclei in May-Grünwald-Giemsa (MGG) staining (unpublished data).

Chymase induces smooth muscle cell apoptosis

To identify the factors responsible for inducing apoptosis of SMCs, we separated the mast cell releasate into granule remnants and granule remnant-free releasate. The granule remnants induced apoptosis of SMCs like the total mast cell releasate, whereas the granule remnant-free releasate had no significant effect (Study I, Figure 1 B and C). We further purified the three main components of the granule remnants, i.e., heparin PGs, chymase, and carbocypeptidase A, and examined their ability to induce apoptosis of SMCs. It was found that, of the total mast cell releasate, only chymase was able to induce apoptosis of SMCs (Study I, Figure 2).

The ability of chymase to induce apoptosis of SMCs was further confirmed by four independent methods (***Study I, Figure 3***). Chymase-treated SMCs showed nuclear fragmentation, as detected by MGG staining, and propidium iodide (PI) staining, and stained positively in TUNEL. In addition, agarose gel electrophoresis of the DNA extracted from SMCs treated with chymase revealed a "ladder" of DNA fragments that consisted of integral multiples of 180-200 base pairs, typical for apoptosis.

Purified chymase induced apoptosis of SMCs in a time- and dose-dependent manner, and apoptosis could be observed as early as 2 hours after exposure to chymase (Study I, Figure 4). Chymase also induced a clear, concentration-dependent decrease in cell numbers in the chymase-treated samples (unpublished data).

Recombinant human chymase induces apoptosis of human coronary artery smooth muscle cells

All the above results and the results to be reported in the subsequent paragraphs of the result section, were obtained by using rat as a model system. To investigate the relevance of these findings in human system, SMCs derived from human coronary arteries (CASMC) were exposed to recombinant human chymase (rh-chymase). Also the rh-chymase was found to induce apoptosis of CASMCs as measured by flow cytometry and confirmed by TUNEL assay (***Study I, Figure 5***).

The ability of chymase to induce smooth muscle cell apoptosis depends on its proteolytic activity

To study whether the proteolytic activity of chymase is responsible for the induction of SMC apoptosis, we exposed SMCs to chymase in the presence of soybean trypsin inhibitor (TRINH), a commercially available serine protease inhibitor and measured apoptosis with FACS. The activity of chymase was measured by N-benzoyl-tyrosine ethyl ester (BTEE) assay. In the presence of 100 μg/mL TRINH the proteolytic activity of purified chymase (20 BTEE units/mL) was fully blocked (0 BTEE units/mL) and the proportion of apoptotic cells (18.4% ± 1.4% in chymase-treated cells) was reduced to the basal level (4.2 ± 1.3% versus 3.2 ± 0.5% in control cells) The TRINH alone was without any effect on SMC apoptosis.

Chymase-mediated apoptosis involves caspase-8

To obtain information about the intracellular mechanisms of chymase-induced apoptosis, SMCs were treated with chymase in the presence of a caspase inhibitor I (Z-VAD-FMK, 100 μmol/L), a broad spectrum inhibitor which has been shown to efficiently inhibit effector caspases, such as caspase-3 (Slee *et al.* 1996; Kawasaki *et al.* 2000) and caspase-7 (Chandler *et al.* 1998). Addition of this inhibitor to the SMCs strongly decreased the chymase-dependent increase in the number of apoptotic cells (***Study I, Figure 6***). We next tested whether inhibiting caspase-8 or -9, two well-known initiator caspases, would have an effect on the chymase-induced apoptosis. Caspase-8 inhibitor reduced the apoptotic rate by about 60%, whereas caspase-9 inhibitor was without effect (***Study I, Figure 6***).

Chymase bound to heparin proteoglycans is able to induce SMC apoptosis in the presence of natural inhibitors

Several natural inhibitors of chymase, such as α_1-antitrypsin, α_2-macroglobulin, and α_1-antichymotrypsin are present in tissue fluids (Travis J *et al.* 1983). In order to study whether chymase-mediated apoptosis can occur under physiological conditions, we incubated SMCs and chymase in the presence of serum. For comparison, several other neutral proteases were also tested for their ability to induce SMC apoptosis under similar conditions. In the absence of serum all the proteases tested induced apoptosis of the growth-arrested rat SMCs (***Study III, Table 1***). However, in serum (10% FCS), only the heparin PG-bound chymase present in exocytosed mast cell granules was able to retain a part (~4%) of its apoptotic potential. Thus, under conditions mimicking those in physiological tissues, i.e., in the presence of protease inhibitors, chymase in its natural form, i.e., when bound to heparin PGs, retained a part of its proteolytic activity (20 vs. 3.8 BTEE units/mL) and was able to induce SMC apoptosis.

Chymase degrades SMC-derived fibronectin

After treating SMCs with proteolytically active chymase, the typical cell spreading of the SMCs (***Study III, Figure 1***) was found to be disturbed and the cells showed a rounded up morphology, suggesting that chymase may induce apoptosis by disrupting their interactions with the extracellular matrix (ECM). Since fibronectin (FN) is known to be an important ECM-component involved in SMC spreading (Hedin *et al.* 1997), and also to be highly susceptible to chymase-mediated proteolysis (Vartio *et al.* 1981), we next

studied the effect of chymase on SMC-derived FN. Untreated SMCs showed extensive cell spreading and positive perinuclear staining of FN, reflecting ongoing synthesis of FN. In addition, a light diffuse positive staining was seen in their pericellular vicinity, indicating secretion of synthesized FN (***Study III, Figure 1 C***). In contrast, SMCs treated with chymase were retracted and showed intense punctate plasma membrane-associated staining of FN (***Study III, Figure 1 D***). Furthermore, the light diffuse staining of pericellular FN observed in the untreated SMCs was lost in the chymase-treated SMCs, indicating extensive degradation of the extracellular FN. By first detaching and removing the cultured SMCs with detergents, we could visualize the SMC-derived intact FN-matrix, which appeared as a typical fibrillar layer attaching to the cover slips on which the SMCs had been cultured (***Study III, Figure 1 C***, inset). In sharp contrast, when such intact FN-matrix was treated with chymase, the fibrillar staining was rapidly lost and intense punctate staining appeared, reflecting extensive degradation of the FN chains (***Study III, Figure 1 D***, inset).

To further analyze the effect of chymase on SMC spreading, we stained the SMCs with an antibody specifically recognizing activated, phosphorylated focal adhesion kinase (p-FAK), a key component in the formation of focal adhesion complexes. Normal spreading of SMCs involves the active formation of focal adhesion complexes, appearing as focal contacts in the plasma membrane of the SMCs (***Study III, Figure 1 E,*** arrows). In contrast, treatment of SMCs with proteolytically active chymase disrupted the focal adhesion complexes, leading to a loss of the focal contacts in the plasma membranes of the SMCs (***Study III, Figure 1 F***). Interestingly, after chymase treatment, p-FAK appeared to accumulate in the nuclei of the treated SMCs (***Study III, Figure 1 F,*** arrows).

Chymase-generated FN degradation products induce SMC apoptosis

To further differentiate between direct effects of chymase and possible additional effects of chymase-generated FN degradation products on SMC apoptosis, we treated commercial plasma-derived FN with heparin PG-bound chymase (granule remnants). The formed FN degradation products were separated from the granule remnants by sedimenting the remnants by centrifugation, after which the aliquots of the protease-free supernatant were added to growth-arrested SMCs. The absence of contaminating active chymase in the FN degradation products was verified by a chromogenic substrate (BTEE), and a highly sensitive reverse-phase high-performance liquid chromatography (RP-HPLC) method (Kokkonen *et al.* 1997). In contrast to untreated intact FN, which had no significant effect on the level of SMC apoptosis, the FN degradation products were found to induce apoptosis in a dose-dependent manner and to a similar extent as chymase (***Study III, Figure 2***).

Chymase and chymase-generated fibronectin degradation products alter the tyrosine phosphorylation status in smooth muscle cells

Since ECM components are known to regulate cell survival through outside-in signaling, i.e., by activating protein tyrosine kinases in the cell survival signaling pathway (Frisch *et al.* 1996), we next studied the effect of chymase and chymase-generated FN degradation products on the tyrosine phosphorylation status in SMCs. Purified chymase induced rapid changes (within 10 min) in the level

of tyrosine phosphorylation in SMCs, and notably, two proteins (~130 kD, ~80 kD) were markedly less phosphorylated (***Study III, Figure 3 A,*** arrows). Furthermore, the chymase-induced SMC apoptosis was completely inhibited (chymase 36 % ±1,5 %, chymase and 500 μM sodium orthovanadate 6,8 % ± 0,45 %, control 5,3 % ± 0,1 %) in the presence of 500 μM sodium orthovanadate, an inhibitor of tyrosine phosphatases, indicating that the chymase-mediated SMC apoptosis is triggered by a mechanism involving tyrosine dephosphorylation. More importantly, the chymase-generated FN degradation products resulted in a dephosphorylation pattern similar to that obtained with chymase, further suggesting that both chymase and the FN degradation products induced SMC apoptosis via a similar outside-in signaling mechanism (***Study III, Figure 3 B***).

Chymase disturbs p-FAK-dependent survival signal transduction cascade

The observed reduction in tyrosine phosphorylation of a 130 kDa molecule (***Study III, Figure 3A***, upper arrow) and the loss of focal adhesion complexes (***Study III, Figure. 1 F***), both suggest that focal adhesion kinase (FAK) itself may be a target of dephosphorylation. In order to test this hypothesis, we treated SMCs with chymase and the FN degradation products and measured FAK and p-FAK protein levels using Western blotting. Both chymase and the FN degradation products induced a time-dependent, strong reduction in the level of phosphorylated active FAK (p-FAK) (***Study III, Figure. 4 A***). However, both chymase and the FN degradation products also induced a similar time-dependent reduction in the protein level of FAK, suggesting an extensive proteolytic degradation of this molecule (***Study III, Figure. 4 A***). The FN degradation products showed a somewhat delayed and less strong effect in FAK degradation, revealing that degradation of pericellular FN by chymase was more efficient in inducing apoptosis than addition of soluble anti-adhesive FN degradation products to cells with intact pericellular FN.

To further verify the inhibition of the p-FAK-dependent signal transduction cascade, we analyzed the degree of phosphorylation and the protein levels of a downstream mediator, Akt. Akt is an important mediator between extracellular signals and modulation of gene expression, metabolism, and cell survival (Scheid *et al.* 2003). We found that both chymase and the FN degradation products (***Study III, Figure 4***, panels C and D) induced Akt dephosphorylation, whereas neither had a significant effect on the level of the Akt protein.

Chymase leads to NFκB degradation and prevents its tranclocation to nucleus

In several cell types, including ECs (Scatena *et al.* 1998; Weaver *et al.* 2002) and neutrophils (Kilpatrick *et al.* 2002), integrin-mediated cell survival signaling has been shown to involve the activation of nuclear factor kappa B (NFκB). In order to test whether the chymase-induced SMC apoptosis involves modulation of the NFκB signaling system, growth-arrested rat arterial SMCs attached to cytoslides were exposed to 3 BTEE units/mL of chymase for 4 hours. The cells were then fixed and stained with an anti-NFκB antibody that recognize the transactivating p65-subunit of NFκB. It was found that both the cytoplasm and the nuclei of the control cells stained weakly for NFκB (***Study V, Figure 1 A***,

upper left, arrows), indicating a moderate constitutive activation and nuclear translocation of NFκB. In contrast, the chymase-treated cells showed no nuclear NFκB staining (upper right, arrows), indicating impaired nuclear translocation. In addition, the chymase-treated cells have a retracted morphology (***Study III, Figure 1 B***) explaining the more intense perinuclear and cytoplasmic staining pattern. To further study the effect of chymase on NFκB activation and nuclear translocation, SMCs were pretreated with chymase for 1.5 hours and then exposed to 5 μg/mL lipopolysaccharide (LPS), a well known activator of NFκB (Müller *et al.* 1993). In the presence of LPS, the nuclear staining was intense (***Study V, Figure 1 A*** lower left, arrows), reflecting NFκB activation and translocation to nuclei. However, pretreating the SMCs with chymase totally abolished the LPS-mediated nuclear translocation of NFκB (***Study V, Figure 1 A*** lower right, arrows). By using Western blotting techniques, we could show that after chymase-treatment (1 hour) the total amount of NFκB, i.e. p65 immunoreactivity, was significantly decreased (see ***Study V, Figure 1 B***), suggesting that the nuclear translocation is lost due to NFκB (p65) degradation.

Similar to the immunhistochemical results, electrophoretic mobility shift assay (EMSA) analysis of nuclear extracts isolated from untreated SMCs showed a low level of constitutive NFκB DNA-binding activity (see ***Study V, Figure 2,*** lane 1). This activity was highly increased when the SMCs were treated with LPS (lane 3). Furthermore, chymase significantly decreased the level of both constitutive and LPS-induced NFκB DNA-binding activity (lane 4). Together these results suggest that chymase, by affecting the total amount of NFκB and thereby its activation and nuclear translocation, disturbs the NFκB-mediated cell signaling system.

Chymase decreases bcl-2 expression

In terms of apoptosis, one of the target genes of NFκB is the antiapoptotic bcl-2, which is involved in SMC apoptosis (Leszczynski *et al.* 1994). To study the effect of chymase on the level and function of the bcl-2 protein, SMCs were cultured on cytoslides, growth-arrested, and exposed to chymase for 16 hours. Untreated SMCs showed a strong punctate immunostaining for bcl-2 (***Study V, Figure 3 A***, upper left), typical for mitochondrial localization. In contrast to bcl-2, bax, a proapoptotic protein from bcl-2 family, seems to reside mainly in the cytoplasm of untreated cells (***Study V, Figure 3 A*** lower left). However, after chymase-treatment bax translocated to the mitochondria as indicated by a more punctate staining pattern (***Study V, Figure 3 A*** lower right), suggesting an activation of the mitochondrial death pathway.

In contrast, treatment with chymase resulted in a loss of the punctate mitochondrial-like staining, leaving only a faint cytoplasmic staining. As bcl-2 in normal cells is mainly localized to mitochondria, these findings suggest a decreased bcl-2 content in mitochondria of chymase-treated cells. To further analyze, whether the loss of mitochondrial bcl-2 staining is a result of decreased bcl-2 gene expression, as could be implicated by the reduced NFκB activation, we measured the level of bcl-2 mRNA in the chymase-treated SMCs as a function of time. The expression of bcl-2 mRNA decreased rapidly after incubation with chymase and was maximally inhibited at 6 hours (See ***Study V, Figure 3 B***).

Chymase activates caspase-3, -8, and -9

Chymase-induced apoptosis could be inhibited with a caspase-8 inhibitor, whereas a caspase-9 inhibitor had no effect (***Study I, Figure 6***). In order to directly study the activation of caspases, we exposed growth-arrested SMCs to chymase for 16 hours and measured the activity of caspase-3, -8, and -9 using specific fluorescent substrates. Chymase did activate both the initiator caspase-8 and -9, and effector caspase-3 (***Study V, Figure 4***). Interestingly, the activity of all 3 caspases was inhibited with both a caspase-8 and a caspase-9 inhibitor, suggesting cross activities between the initiator caspases.

Mast cells and endothelial cell apoptosis

Mast cell activation downregulates bcl-2 expression in cocultured endothelial cells

ECs were isolated from the vasculature of rat myocardium and co-cultured for 4 hours with either resting or activated rat serosal mast cells. In the absence of mast cells, as well as in the presence of resting mast cells (***Study IV Figure 1***, lanes 1 and 3), the ECs expressed significant amounts of mRNA for two genes known to be involved in the regulation of apoptosis, the bcl-2 and the bax. When the mast cells were activated with the mast cell-specific noncytotoxic stimulant compound 48/80, an immediate release of ~80% of the stored histamine ensued, reflecting significant mast cell degranulation. Within 30 minutes of the degranulation, down-regulation (82%) of the mRNA expression of bcl-2 was observed in co-cultured ECs (***Study IV, Figure 1,*** lane 2, top panel). In contrast, the expression level of bax (***Study IV, Figure 1*** lane 2, middle panel) was not significantly affected. These results show that mast cell degranulation specifically down-regulated bcl-2 expression in the co-cultured ECs.

To further identify the component(s) in the material released from the activated mast cells, i.e., mast cell releasate, which is (are) responsible for the observed down-regulation of bcl-2 mRNA expression, we separated the releasate into granule remnants and a granule remnant-free supernatant by centrifugation at 13,000*g* for 10 min. The major effect of bcl-2 down-regulation was found to reside in the sedimented granule remnant fraction (***Study IV, Figure 2 A,*** top panel). In addition, a smaller effect was also found to be present in the granule remnant-free supernatant, whereas neither of the two fractions had a significant effect on the mRNA expression of bax (***Study IV, Figure 2 A,*** middle panel). By using competitive RT-PCR, the granule remnant-mediated down-regulation of the bcl-2 mRNA expression was found to be rapid, being already maximal (10-fold) after incubation for 30 minutes (***Study IV, Figure 2 B,*** top panel) and the inhibitory effect lasted for up to 8 hours. Furthermore, there were no significant changes in the expression level of bax (***Study IV, Figure 2 B,*** middle panel). To verify the results obtained with RT-PCR, we analyzed the protein levels of bcl-2 and bax with western blotting in ECs, which were treated with granule remnants. As shown by immunoblotting, incubation of the ECs for 4 hours in the presence of granule remnants (***Study IV, Figure 3***, upper panel) resulted in a significant reduction in bcl-2 protein. No significant change in the protein level of bax was observed even after a prolonged period (18 h) of granule remnant treatment (***Study IV, Figure 3,*** lower panel).

Mast cell granule remnants induce changes in bcl-2 and cytochrome c localisation in endothelial cells

Both the concentration and the localization of bcl-2 and bax in mitochondria are important for the integrity of the mitochondria. Since changes in these two parameters are associated with mitochondrial leakage and onset of apoptosis, we analyzed their subcellular localization by immunocytochemistry. Untreated ECs exhibited a strong punctate immunostaining of bcl-2 in their cytoplasm (***Study IV, Figure 4,*** Panel A), compatible with the typical localization of bcl-2 to mitochondria. In addition, the cell nuclei also stained strongly for bcl-2. However, treatment of ECs with mast cell granule remnants resulted in extensive loss of the observed punctate immunostaining for bcl-2 (***Study IV, Figure 4,*** Panel B), reflecting a decreased bcl-2 content in the mitochondria. The staining pattern for bax was found to be similar to that for bcl-2 (***Study IV, Figure 4,*** Panel C), indicating co-localization of the two apoptosis-related bcl-2 family members. However, in contrast to bcl-2, bax appeared to remain localized to the mitochondria even after treatment with granule remnants (***Study IV, Figure 4,*** Panel D). Furthermore, untreated ECs also showed a positive punctate immunostaining for cytochrome c in their mitochondria (***Study IV, Figure 4,*** Panel E), whereas the granule remnant-treatment resulted in a more diffuse staining of the cytoplasm (***Study IV, Figure 4,*** Panel F). This result suggests that, in the presence of mast cell granule remnants, cytochrome c, a known initiator of caspase activation if present in the cytoplasm, was released from mitochondria into the cytoplasm of ECs.

To further verify the translocation of cytochrome c from mitochondria to the cytoplasm, we subjected ECs to subcellular fractionation and analyzed the level of cytochrome c in the two specific subfractions. The results of this experiment confirmed that treating ECs with mast cell granules resulted in a translocation of cytochrome c from the mitochondria into the cytoplasm (***Study IV, Figure 5***), indicating a role for mitochondria leakage and cytochrome c release in the onset of mast cell-mediated EC apoptosis.

Mast cell granule remnants induce endothelial cell apoptosis

The apoptotic manifestation of the mast cell-mediated down-regulation of bcl-2 expression was further verified by propidium iodide- (PI), Hoechst 33342-, and Annexin-V-FLUOS staining, and flow cytometric analysis of ECs treated with mast cell-derived granule remnants. PI- and Hoechst 33342-staining of granule remnant-treated ECs showed signs of nuclear fragmentation, chromatin condensation, and blebbing typical for apoptotic cells (***Study IV, Figure 6***). Annexin-V-FLUOS staining further showed the presence of phosphatidyl serine on the surface of the treated ECs. Furthermore, by analyzing the granule remnant-treated ECs with flow cytometry, we could detect and quantitate a subgenomic DNA peak consistent with the presence of a cell population with apoptotic features. In addition, agarose gel electrophoresis of DNA isolated from granule remnant-treated ECs showed a ladder of DNA fragments (~180 base pair increments) typical for apoptotic cells. In a parallel experiment, we excluded the presence of significant necrosis by demonstrating that the culture media were devoid of lactate dehydrogenase (unpublished data).

Mast cell-mediated endothelial apoptosis involves TNF-α

To further define the compound(s) responsible for the observed proapoptotic effects, we incubated ECs separately with three granule remnant components that are capable of inducing apoptosis, i.e., chymase, TGF-β, and TNF-α. We found that the mRNA expression of bcl-2 was significantly down-regulated (60%) in the presence of TNF-α, suggesting that TNF-α present in the mast cell granule remnants is involved in the observed effect (***Study IV, Figure 7 A***). Finally, to directly demonstrate that TNF-α is involved in granule remnant-mediated EC apoptosis, we incubated ECs with granule remnants in the presence of a TNF-α–neutralizing antibody. We found that in the presence of a TNF-α–neutralizing antibody, the granule remnant-induced EC apoptosis was partially inhibited (***Study IV, Figure 7 B***).

Mast cell chymase inhibits smooth muscle cell growth and collagen expression

Mast cell chymase inhibits smooth muscle cell proliferation by blocking the $G_0/G_1 \rightarrow S$ transition

To study the effect of chymase on SMC growth, we incubated growth-arrested SMCs with increasing concentrations of chymase and measured incorporation of [^{3}H]thymidine after adding FCS to release cells from growth arrest. Incorporation of [^{3}H]thymidine by growth-arrested SMCs was strongly inhibited in the presence of increasing amounts of chymase activity (***Study II, Figure 1 A***). In addition, counting the cells at the end of the incubation revealed a dose-dependent decrease in the cell number (***Study II, Figure 1 B***). When growth-arrested SMCs were preincubated with mast cell chymase (20 BTEE units/mL) for 16 hours, we observed a small reduction in cell number (***Study II, Figure 1 D***, 0 time point) which was due to chymase-mediated apoptosis and disruption of cell-matrix interactions with ensuing loss of adhering SMCs. Interestingly, when FCS was added to the incubation medium to inhibit chymase activity and to stimulate SMC proliferation, the rate of incorporation of [^{3}H]thymidine by the SMCs was found to be significantly reduced (***Study II, Figure 1 C***). The chymase-mediated growth-inhibitory effect lasted for up to 72 hours, after which there was a rapid increase in [^{3}H]thymidine incorporation to a level comparable to that of untreated cells. A similar effect was observed in the number of SMCs (***Study II, Figure 1 D***). In a parallel experiment, we found only low percentages of apoptotic cells (analyzed by flow cytometry) during the exponential growth phase: about 2% in the chymase-treated SMC monolayer and 0.4% in the untreated cells. Moreover, only a few trypan blue-positive cells were observed in either the chymase-treated or the untreated SMC monolayers. By using flow cytometric analysis, we found that the proportion of SMCs in the G_0/G_1 phase was significantly higher in the presence of chymase (54.6±2.5% versus 47.3±0.9% for no treatment). In addition, the proportion of cells in the S phase was significantly lower in the presence of chymase (32.1±3.0% versus 38.0±0.7% for no treatment), suggesting inhibition or delay of the $G_0/G_1 \rightarrow S$ transition in the chymase-treated SMCs.

Anti-TGF-β neutralizing antibody partially blocks the inhibitory effect of preconditioned medium derived from chymase-treated smooth muscle cells

Because SMCs are known to produce and secrete TGF-β1 avidly and to respond to it by changing their rates of growth (Bobik *et al.* 1999), we tested whether TGF-β1 was involved in the observed chymase-mediated inhibition of the growth. In the absence of anti-TGF-β neutralizing antibodies the preconditioned medium induced a 50% decrease in the rate of [^{3}H]thymidine incorporation and in the cell number (***Study II, Figure 2***). In the presence of increasing concentrations of of anti-TGF-β neutralizing antibody, the rate of [^{3}H]thymidine incorporation and cell number correspondingly increased, suggesting that TGF-β1 was responsible for the chymase-mediated inhibition of SMC growth. Addition of increasing concentrations of an anti-TGF-β antibody that recognizes active form of TGF-β to control SMCs did not increase their growth rate (unpublished data), indicating that the observed effect was due to the presence of active TGF-β1 in the preconditioned medium. Furthermore, similar results were obtained with equivalent amounts of recombinant human TGF-β1. (***Study II, Figure 2 C and D***) Interestingly, inhibition of chymase activity in the preconditioned medium completely abolished the observed effects on DNA synthesis and cell growth in the treated SMCs (***Study II, Figure 2 C and D***).

Mast cell chymase releases active TGF-β1 from extracellular matrix of smooth muscle cells

To verify that rat aortic SMCs produce TGF-β1, we analyzed SMC monolayers by immunocytochemistry. SMCs were found to stain positively with a polyclonal antibody that recognizes both active and latent forms of TGF-β1 (***Study II, Figure 3 A***). To separate intra- and extracellular TGF-β1, SMCs were cultured for 48 hours, after which the cells were gently removed with 0.5% sodium deoxycholate. The remaining SMC-derived ECM also stained positively for TGF-β1.

As a further test of whether mast cell chymase could release and activate the TGF-β1 secreted by rat aortic SMCs, the cell monolayers were incubated with increasing amounts of chymase, and the concentration of TGF-β1 in the culture medium was detected by immunoblotting. The amount of active TGF-β1 (25 kDa) in the culture medium gradually increased in the presence of increasing concentrations of chymase. (***Study II, Figure 3 B***) By performing an ELISA assay with TGF-β1 type II serine/threonine kinase receptor (TβRII) as capture, we found that chymase significantly increased the amounts of biologically active TGF-β1 released from the SMC monolayer (282±85 versus 653±77 pg/10^6 cells) and from the SMC-derived ECM (0 versus 140±28 pg/10^6 cells). Futhermore, the expression of PAI-1, an indicator of active TGF-β1, was significantly induced (4.6 fold) in the presence of chymase (***Study II, Figure 4A***). No effect was observed in the expression of TβRI or TβRII. In contrast, in the chymase-treated SMCs, the ratio (target/competitor) of the specific TGF-β1 signal was decreased (2.4 fold) (***Study II, Figure 4B***).

Mast cell chymase inhibits the expression of collagen type I and type III in a TGF-β-independent manner

Physiologically active TGF-β1 is known to induce collagen expression in SMCs *in vitro* (Amento *et al.* 1991). To test whether

mast cell chymase can induce collagen synthesis by SMCs through its activation of ECM-bound latent TGF-β1, the expression of mRNA of collagen types I and III, the predominant forms of collagen in atherosclerotic lesions (Murata *et al.* 1986), were detected by competitive RT-PCR. Surprisingly, incubation of SMCs with chymase resulted in reduced mRNA expression of collagen type I (69 %) and collagen type III (79 %) (***Study II, Figure 5 A***). Furthermore, addition of an anti-TGF-β neutralizing antibody to chymase-containing culture medium did not block the inhibitory effect of chymase on collagen expression in SMCs (***Study II, Figure 5 B***). These results suggest that the observed chymase-mediated inhibition of collagen mRNA expression had occurred independently of chymase-mediated TGF-β1 activation. Indeed, the preconditioned medium, which was found to contain about 2 ng/mL of active TGF-β1, had no effect on the collagen expression in the SMCs. However, when SMCs were treated with high concentrations of rhTGF-β1 (200 ng/mL), the expression of collagen type I was increased (2-fold), as has also been reported earlier (Amento *et al.* 1991). Taken together, the above results are compatible with the notion that the chymase-induced decrease in SMC collagen expression did not depend on the presence of active TGF-β1. Furthermore, incubation of human coronary arterial SMCs with recombinant human chymase for 48 hours resulted in reduced expression of collagen I (74 %) and collagen III (23 %) in the treated human SMCs (***Study II, Figure 5 C***).

DISCUSSION

Erosion or rupture of an atherosclerotic plaque with ensuing thrombosis is the cause of majority of all coronary events (Falk 1992; Lee *et al.* 1997). Inflammatory cells, such as macrophages, T-cells and mast cells, being able to induce ECM degradation, loss of endothelium, inhibition of collagen production, and induction of cell death, may contribute to plaque disruption (Libby 2002). Increased numbers of mast cells have been observed at sites of plaque erosion or rupture in patients that have died of acute myocardial infarction (Kovanen *et al.* 1995), raising the hypothesis that mast cells could be involved in plaque destabilization. In order to test this hypothesis, we investigated the effects of mast cell-derived products on the growth, collagen synthesis, and death of intimal SMCs and ECs *in vitro*.

Mechanism of chymase-induced smooth muscle cell apoptosis

The present study showed that activated rat serosal mast cells induce apoptosis of rat aortic SMCs and identified chymase, a neutral serine protease secreted by the activated mast cells, as the active proapoptotic component. In addition, recombinant human chymase was found to be capable of inducing significant apoptosis in cultured human coronary artery SMCs, indicating that the observation is not restricted to rodents, but is also potentially relevant in humans. The chymase-mediated apoptotic effect was completely dependent on the proteolytic activity, since inhibition of chymase with a trypsin inhibitor (TRINH) fully abolished the induction of SMC apoptosis. Also, serum, which contains natural protease inhibitors, notably members of the serpin superfamily (Travis J *et al.* 1983) completely blocked the proteolytic activity of purified chymase, and thus, prevented it from inducing apoptotic death of SMCs.

However, chymase may be unique in that, after being secreted into the extracellular fluid, it remains bound to the heparin PG matrix of the granule remnants and retains a part of its activity even in the presence of natural protease inhibitors, such as α_1-antitrypsin, α_2-macroglobulin, and α_1-antichymotrypsin (Lindstedt *et al.* 2001a). To mimick the conditions present in the extracellular fluid of tissues, we have tested several neutral proteases for their ability to induce apoptosis in the presence of serum. Among the proteases tested, only heparin PG-bound chymase retained a part (~19 %) of its proteolytic activity in the presence of natural protease inhibitors (10% FCS), and was capable of inducing apoptosis. Thus, in comparison to other neutral proteases, mast cell-derived granule remnant-bound chymase may possess the ability to degrade ECM components under *in vivo* conditions.

The protease-induced apoptosis is not a unique phenomenon for mast cell chymase and SMCs, as several other neutral proteases have been shown to exert proapoptotic effects upon various cell types. The neutrophil serine proteases, elastase and proteinase 3, induce apoptosis in ECs (Yang *et al.* 1996), α-chymotrypsin and trypsin induce apoptosis in neutrophils (Trevani *et al.* 1996), and rat mast cell chymase induce apoptosis in cardiomyocytes (Hara *et al.* 1999). Moreover, granzymes, serine proteases expressed exclusively by cytotoxic T lymphocytes and natural killer cells, are able to enter the target cells via perforin-mediated

endocytosis and trigger apoptosis by directly activating caspases (Trapani 2001). Although granzymes, in their cleavage specificity, show similarities with chymase (Kam *et al.* 2000), the granzyme-mediated apoptosis differs from the chymase-mediated apoptosis, as chymase exerts its proapoptotic activity in the extracellular space by affecting the ECM-cell interactions.

In this study, we have identified the ECM component fibronectin (FN) to be a highly susceptible target of chymase-mediated proteolysis and a crucial trigger of mast cell-mediated SMC apoptosis (Study III). Moreover, addition of purified chymase-generated FN degradation products to SMCs in culture induced SMC apoptosis similar to that observed with chymase. We also observed that the FN degradation products induced similar outside-in tyrosine phosphorylation signaling in SMCs, as did chymase. In particular, the focal adhesion kinase (FAK), one of the key mediators of focal adhesions and ECM-cell interactions was rapidly degraded, i.e., inactivated, in the presence of chymase and/or FN degradation products, leading to dephosphorylation of Akt, a downstream mediator of the FAK-dependent survival signaling cascade. Moreover, chymase-induced SMC apoptosis could be inhibited by sodium orthovanadate, an inhibitor of tyrosine phosphatases, showing that changes in the stage of phosphorylation is critical in chymase-induced apoptosis.

The ECM exerts profound control over cells, regulating their proliferation, differentiation and apoptosis. These effects are primarily mediated by the integrins, which regulate the activities of various cytoplasmic kinases, growth factor receptors, and ion channels (Giancotti *et al.* 1999). The major FN-binding integrin, α5β1, has also been shown to mediate cell survival through outside-in signaling by activating protein tyrosine kinases, notably the FAK (Frisch *et al.* 1996; O'Brien *et al.* 1996; Matter *et al.* 1998). Interestingly, chymase and chymase-generated FN degradation products, when added to fresh cultures of SMCs, induced rapid degradation of FAK, suggesting that chymase interferes with the FN-α5β1 integrin signaling pathway in the SMCs.

In several cell types, including ECs (Scatena *et al.* 1998; Weaver *et al.* 2002) and neutrophils (Kilpatrick *et al.* 2002), integrin-mediated cell survival signaling has been shown to involve the activation of nuclear factor κB (NFκB). NFκB regulates numerous genes involved in inter- and intracellular signaling, cellular stress responses, cell growth, differentiation, and survival (Gerondakis *et al.* 1999; Pahl 1999). Chymase treatment reduced the total amount of NFκB and abolished the translocation of active NFκB (p65) to the nucleus (Study V). A loss of NFκB-mediated transactivation resulted in a downregulation of bcl-2 mRNA, followed by reduced amounts of bcl-2 in the mitochondria. This is in accordance with the idea that NFκB is responsible for the expression of antiapoptotic molecules, such as bcl-2. These results suggest that chymase is able to interfere with the NFκB-mediated cell survival signaling system in SMCs.

Previously, two separate anti-apoptotic pathways associated with FN-integrin interactions and FAK-mediated downstream signaling have been demonstrated. Firstly, after serum withdrawal, FN has been shown to promote survival signals in fibroblasts and ECs by suppressing p53-mediated apoptosis through the FAK,

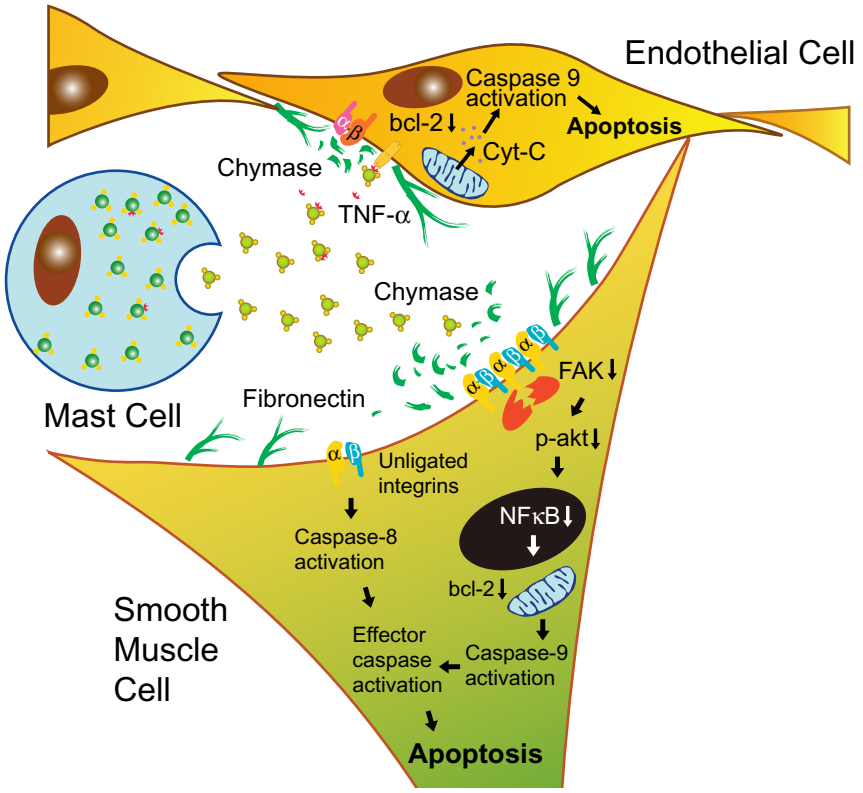

Figure 7. Proposed mechanisms of mast cell-induced apoptosis

A schematic model illustrating the proposed mechanisms of mast cell induced apoptosis in SMCs and ECs. In SMCs, mast cell chymase degrades FN, disturbing the FN-α5β1 integrin interaction with subsequent degradation of FAK and loss of focal adhesions. As a consequence, Akt is dephosphorylated and NFκB is degraded, leading to downregulation of antiapoptotic bcl-2. In ECs, TNF-α released from activated mast cells downregulates bcl-2, leading to cytochrome c release and apoptosis.

Ras/rac1/Pak1/MAPK kinase 4 (MKK4), and c-Jun NH2-terminal kinase (JNK) pathways (Almeida *et al.* 2000). Secondly, binding of the α5β1 integrin to FN in CHO cells upregulates the expression of anti-apoptotic genes, such as bcl-2, through an FAK-SHC-Ras-phosphatidylinositol 3´-kinase (PI3-kinase)-Akt pathway (Matter *et al.* 2001). Our present results show similarities with the latter pathway, in that chymase is able to disrupt focal adhesion-dependent NFκB-mediated survival signaling (Fig 7).

The final execution of the apoptotic process, i.e. the point of no return, is the activation of caspases. Treatment of SMCs with chymase induced activation of the initiator caspases -8 and -9, followed by activation of the executor caspases-3. Although, our initial results indicated that caspase-8 was the major pathway in chymase-mediated SMC apoptosis (Study I), our latter results have suggested an additional role for caspase-9 in this process. Thus, in contrast to previous data (Budihardjo *et al.* 1999), clearly distinguishing between caspase-8, being the initiator of death receptor (extrinsic) pathway, and caspase-9, being the initiator of the mitochondrial (intrinsic) pathway of apoptosis, an increasing amount of studies, including our own, suggest a close interplay between the two cascades (Lassus *et al.* 2002). For instance, the

mitochondrial pathway is used as an amplification loop for the death receptor pathway (Li *et al.* 1998). Thus, the initiator caspases may interact and amplify each other in the process of activating the executor caspases. Moreover, caspase-2 has recently emerged as the most proximal caspase activated in cell stress signaling, and is able to activate both intrinsic and extrinsic apoptotic pathways (Schweizer *et al.* 2003; Troy *et al.* 2003). Presently, we do not know whether caspase-2 is involved in chymase-induced SMC apoptosis. Recent data, showing that caspase-8 may be activated by unligated integrins (Stupack *et al.* 2001) further suggest that there may also be additional pathways capable of triggering activation of apoptosis. Our present study, showing disruption of focal adhesions, and hence, creation of unligated integrins, may involve the described mechanism of integrin-mediated caspase-8 activation.

Interestingly, cells seem to differ in their susceptibility to undergo protease-induced apoptosis, as peripheral blood monocytes, lymphocytes, several human leukemic cell lines (THP-1, HL-60, K562), murine L929 fibroblasts, mouse peritoneal macrophages (Trevani *et al.* 1996) and myocardial fibroblasts (Hara *et al.* 1999) fail to undergo apoptosis after treatment with proteases. Many of these cell types (monocytes, lymphocytes and leukemic cell lines) grow in suspension and, consequently, do not depend on anchoring to ECM for their survival, partly explaining their resistance to protease-induced apoptosis. In contrast, fibroblasts and macrophages typically adhere to various components of the ECM. The mechanisms of these cells against protease-induced apoptosis remain to be clarified. Importantly, the mast cell-mediated proapoptotic effect seems to be a purely paracrine phenomenon, since activated mast cells themselves survive the process of degranulation by overexpressing the prosurvival bcl-2 homologue A1 (Karsan *et al.* 1996).

Taken together, the present findings suggest an additional mechanism by which apoptosis of SMCs might be triggered in atherosclerotic lesions, namely, the action of chymase secreted by activated mast cells.

Mast cell -induced endothelial cell apoptosis

Activated mast cells can also induce EC apoptosis by a mechanism involving rapid down-regulation of bcl-2 expression followed by the release of cytochrome c from the mitochondrial compartment (Study IV). The mast cell-mediated apoptotic effect was found to depend on mast cell stimulation and degranulation and to be present in the granule remnants, and, to a lesser degree, also in the granule remnant–free supernatant. Furthermore, the observed apoptotic effect was partially inhibited with a TNF-α neutralizing antibody, revealing involvement of TNF-α in this process. TNF-α has previously been shown to induce apoptosis in ECs by a mechanism involving down-regulation of the antiapoptotic molecule bcl-2 (Rossig *et al.* 2000).

Both the granule remnants and the granule remnant-free supernatant were capable of down-regulating bcl-2 mRNA expression in ECs, suggesting that mast cells may exert both short-range (granule remnant-bound TNF-α) and long-range (soluble TNF-α) paracrine TNF-α-mediated effects. Whether there are other functional differences between the two fractions, such as differences in terms of TNF-α presentation, is presently not known. In addition to the preformed TNF-α released upon degranulation, stimulated mast cells

have also been shown to secrete newly synthesized TNF-α (Gordon *et al.* 1991), a process that occurs more slowly but may enhance the initial response obtained with the rapidly secreted preformed TNF-α. Thus, our present results suggest that stimulated and degranulated mast cells, as a source of preformed granule remnant-bound and soluble TNF-α, as well as newly synthesized soluble TNF-α, may have both acute and sustained effects on ECs.

Cultured ECs are normally highly resistant to apoptosis (Pohlman *et al.* 1989; Bach *et al.* 1997) and concomitant inhibition of either RNA- or protein synthesis is needed to trigger the apoptotic mechanisms induced by various factors, including TNF-α (Pohlman *et al.* 1989). Thus, in the presence of TNF-α, ECs protect themselves by activating the NFκB-transcription factor pathway, which induces several anti-inflammatory and antiapoptotic pathways, including bcl-2 (Bach *et al.* 1997; de Moissac *et al.* 1999). Interestingly, in our study the observed down-regulation of the bcl-2 mRNA expression level in the ECs was found to be more extensive with mast cell granule remnants than with purified recombinant TNF-α. Thus, it is likely that the granule remnant–mediated apoptosis in the cultured ECs is primed and supported by additional proapoptotic components present in the granule remnants, such as chymase (Hara *et al.* 1999). Like SMCs, also ECs require intact ECM components, notably vitronectin (VN) for their survival (Scatena *et al.* 1998). As chymase is known to degrade VN, in addition to FN (Mayr *et al.* 2001), it is possible that VN degradation is involved in EC apoptosis.

The mast cell mediated SMC and EC apoptosis share mechanistical similarities, in that integrin mediated outside-in signaling is crucial for cell survival. However, mast cell mediated endothelial cell apoptosis seems to involve also the secretion of proapoptotic cytokines, such as TNF-α.

Mast cells as proapoptotic cells

Owing to the strategic location of mast cells at the host-environment interface and their extremely potent repertoire of mediators, mast cells have been proposed an evolutionary role in primary host defense, i.e., in killing invading bacteria and parasites, as well as infected and damaged cells (Gallagher *et al.* 2003; Puxeddu *et al.* 2003). Since a "clean" process of apoptotic death, in contrast to "dirty" necrotic death, would be the most feasible and physiological mechanism of such host defense, one could reason that mast cell-mediated proapoptotic mechanisms could exist as a part of the normal host defense. Indeed, our results demonstrate that mast cells, by releasing chymase and TNF-α, are able to induce SMC and EC apoptosis *in vitro.* In accordance, mast cell- derived chymase has previously been reported to induce cardiomyocyte apoptosis (Hara *et al.* 1999). Moreover, a recent paper suggested that mast cells are able to trigger apoptosis of Jurkat T leukemia cells through caspase-3, -8 and –9 independent mechanisms (Gallagher *et al.* 2003). Thus, mast cells appear to possess several independent proapoptotic mechanisms, and these may be cell-specific.

Prerequisites for mast cell-mediated apoptosis

All current reports on mast cell- mediated apoptosis derive from *in vitro* studies, with no *in vivo* confirmation. Thus, the definite proof of the relevance of mast cell- mediated apoptosis in atheroscle-

rosis awaits future studies. However, the prerequisites for mast cell- mediated apoptosis seem to be fulfilled in atheromas. Thus, in order to exert their effects mast cells have to be present in atherosclerotic lesions, and futhermore, they need to become activated. Recent studies have demonstrated the presence of activated, degranulated mast cells in human coronary plaques (Kaartinen *et al.* 1994a; Laine *et al.* 1999). Mast cell activation is normally strictly regulated, and normal arteries show low level of mast cell activation (Kaartinen *et al.* 1994a). Inflammatory mechanisms, such as complement activation, could be involved in mast cell activation in atheromas (Laine *et al.* 2002). Indeed, in the ruptured coronary plaques activated mast cells were associated with other inflammatory cells (Laine *et al.* 1999). It is possible that under chronic pathological conditions, such as atherogenesis, the ongoing mast cell activation may contribute to undesired and excessive tissue and cell destruction.

Another important question is whether the number of mast cells in atheromas is high enough to influence the neigboring cells. Interestingly, the mast cell to SMC ratio has been determined to be 1:5 at the immediate site of erosion and rupture (Kovanen *et al.* 1995), which corresponds to the cell numbers used in our *in vitro* studies. Moreover, the concentration of chymase has been estimated to be 0.6 μg/mL in the intimal fluid of such vulnerable areas (Lindstedt *et al.* 2001a), which is equivalent to approximately 6 BTEE units/mL, a concentration capable of inducing apoptosis *in vitro*.

Mast cells inhibit smooth muscle cell growth and collagen expression

In addition to SMC and EC apoptosis, the plaque stability is affected by the rate of SMC proliferation and collagen synthesis. In the present study, we have described a novel mast cell chymase-dependent, TGF-β1-mediated paracrine growth-inhibitory effect on SMCs. By adding purified chymase to cultured rat aortic SMCs of synthetic phenotype we could obtain active soluble TGF-β1, which was capable of inhibiting their proliferation by blocking the G0/G1 →S cell cycle transition. Furthermore, chymase-mediated activation of TGF-β1 induced the expression of PAI-1, a sensitive bioindicator of TGF-β1-mediated cellular responses (Yang *et al.* 1999; D'Angelo *et al.* 2001), in the SMCs. Thus, we hypothesize that activated mast cells, by releasing chymase, may inhibit SMC growth through activation of TGF-β1 in tissues in which mast cells and SMCs coexist.

Interestingly, mast cell chymase, although releasing active TGF-β1 (a known collagen-producing factor) from the ECM of the SMCs, has a net inhibitory effect on the expression of collagens of type I and type III in vascular SMCs. Indeed, under *in vitro* conditions, TGF-β1, depending on its concentration, was either without effect or increased the expression of type I collagen by SMCs. Therefore, we infer that the inhibitory effect of chymase on collagen mRNA expression in the SMC cultures was not TGF-β1 dependent. Since only active chymase exerted the inhibitory effect, the inhibitory mechanism(s) is likely to involve proteolytic cleavage of some extracellular factor(s) rather than mere binding of chymase to cell surface structures. As SMCs are the sole producers of the tensile components of the ECM (notably of collagen type I), a decrease in their numbers and synthesizing activity would lead to diminished production of these plaque-stabilizing molecules.

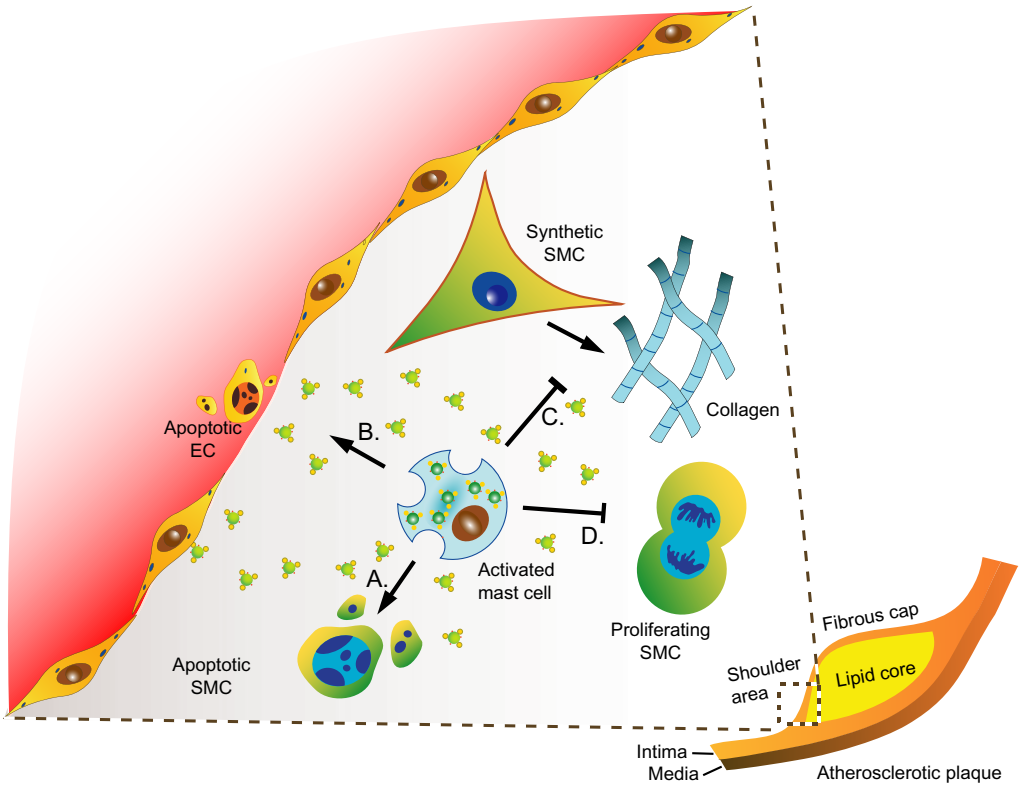

Figure 8. A schematic model illustrating the various effects of activated intimal mast cells in the pathological process of weakening and rupture of an atherosclerotic plaque.

The model depicts how activated mast cells in the shoulder region (the magnified area) of an atherosclerotic plaque may **A.)** induce SMC apoptosis, **B.)** induce EC apoptosis, **C.)** inhibit SMC-mediated collagen synthesis, and **D.)** inhibit SMC proliferation.

Mast cells may destabilize coronary plaques

Taken together, our findings raise the possibility that mast cells could participate in destabilization of coronary plaques by affecting the growth, function, and death of intimal SMCs and ECs. As shown in Figure 8, activated mast cells may induce SMC apoptosis by releasing chymase (A), induce EC apoptosis by releasing TNF-α (B), inhibit collagen expression by SMCs (C), and inhibit SMC proliferation (D). When the present results are interpreted in the light of previous studies, showing that mast cells can inhibit proliferation of rat SMCs *in* vitro by releasing heparin PGs (Wang *et al.* 1999) and synthesize and activate matrix metalloproteinases (Di Girolamo *et al.* 2000), activated intimal mast cells appear to be a highly potential source of molecules capable of affecting plaque remodeling. However, the role of other inflammatory cells in the process of plaque weakening and rupture should not be underscored. In this context, the mast cell-mediated remodeling of the plaque, as described above, serves as a model for the overall role of inflammatory cells in the pathological process of plaque rupture. Future experiments will be necessary to determine the importance of the individual types of inflammatory cells in the onset, progression and execution of the pathological processes ultimately culminating in the rupture of an atherosclerotic plaque.

CONCLUSIONS

The aim of this study was to examine the possible role of mast cells in inducing apoptosis of SMCs and ECs. The following results were obtained in our *in vitro* studies.

1. Chymase released from activated rat serosal mast cells is capable of inducing apoptosis of rat aortic SMCs *in vitro.* The proapoptotic effect requires proteolytic activity of chymase and is time- and dose- dependent. In addition to rat chymase, recombinant human chymase induces apoptosis of human coronary arterial SMCs in culture.

2. The mechanism of chymase-induced apotosis involves degradation of an ECM component, fibronectin (FN). Commercial FN degraded by chymase induces SMC apoptosis in a similar magnitude, and with similar changes in outside-in signaling as chymase. FN degradation is followed by disruption of focal adhesions and degradation of focal adhesion kinase (FAK), one of the key mediators of integrin-ECM interactions and cell survival. Loss of phosphorylated FAK (p-FAK) leads to a rapid dephosporylation of the p-FAK-dependent downstream mediator, Akt. Dephosphorylation appears to be critical for the chymase-induced apoptosis, as tyrosine phosphatase inhibitor sodium orthovanadate prevents the proapoptotic effect.

3. Following chymase treatment, the total amount of nuclear factor kappa B (NFκB) was reduced and the translocation of active NFκB (p65) to the nucleus was abolished. Loss of NFκB-mediated transactivation resulted in downregulation of bcl-2 mRNA expression. Due to the lack of antiapoptotic bcl-2, the apoptotic process was initiated and further executed by the activation of procaspases -3, -6, -8 and -9.

4. Mast cell activation induces apoptosis of cocultured ECs. The effect is partially dependent on the presence of tumor necrosis factor-α and involves bcl-2 downregulation and release of cytochrome c from mitochondria into the cytoplasm.

5. Mast cell chymase inhibits SMC growth and collagen expression through both transforming growth factor-β dependent and independent mechanisms.

ACKNOWLEDGMENTS

The research for this thesis was carried out at the Wihuri Research Institute during the years 1996 - 2003. This has been a great time and I am very grateful to my coworkers and friends for making it so. The Wihuri Research Institute has been extremely flexible, allowing me to divide my time between research, medical studies, international activities and clinical work. I have been priviledged to be able to follow so many interests at the same time.

I wish to express my gratitude for the contribution of the following people:

Professor Petri Kovanen, head of the Wihuri Research Institute and supervisor of my thesis. Petri recruited me to Wihuri, taught me the basics of science and provided the facilities to carry out the research for the thesis. His advice has been invaluable, both *in vitro* and *in vivo*.

Docent Ken Lindstedt, the other supervisor of my thesis and my mentor. Ken provided the leadership and organizational skills, as well as superb methodological knowledge that made the progression of my thesis possible. He taught me to write scientifically, introduced me to several outstanding wines and has been a good friend.

Doctor Yenfeng Wang, a coauthor in all my papers, introduced me to cell culture and several laboratory techniques, and provided a model of an outstanding scientist.

Professor Dariusz Leszczynski introduced me to flow cytometry and helped to overcome the initial problems of apoptosis research.

Doctor Naotaka Shiota, a coauthor in two of my papers, taught me the importance of paying attention to details in scientific work.

Illustrations of Sole Lätti, a coauthor in one of my papers, were a source of inspiration when I started to draw myself.

Monica Schoultz, Jaana Tuomikangas and Mari Jokinen made the progression of the work possible with their experience and technical assistance.

Riina Oksjoki read through the manuscript and improved the language.

Laura Fellman efficiently organized the practical matters.

Docent Hannu Järveläinen and professor Veli-Pekka Lehto reviewed this manuscript and provided valuable comments and constructive criticism.

The staff of Wihuri Research Institute provided help, friendship and entertaining discussions during coffee breaks, not to forget some outstanding parties. Especially I want to acknowledge Mikko Mäyränpää , Suvi Mäkinen and my roommate Inka Liesmaa.

My mother Marja introduced me to science and shares my curiosity in what makes the world tick. My love for printed word is inherited from my father Timo, whose library I started to ransack at early age.

Jaakko and Mikko, my closest friends, are acknowledged for their encouragement and friendship, as well as providing down to earth feedback.

Liina, for everything.

References

Almeida EA, Ilic D, Han Q, Hauck CR, Jin F, Kawakatsu H, Schlaepfer DD, Damsky CH. Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase. *J Cell Biol* 149: 741-754, 2000.

Amento EP, Ehsani N, Palmer H, Libby P. Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler Thromb* 11: 1223-1230, 1991.

Anderson TJ. Nitric oxide, atherosclerosis and the clinical relevance of endothelial dysfunction. *Heart Fail Rev* 8: 71-86, 2003.

Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 19: 2499, 1991.

Arbustini E, Dal Bello B, Morbini P, Burke AP, Bocciarelli M, Specchia G, Virmani R. Plaque erosion is a major substrate for coronary thrombosis in acute myocardial infarction. *Heart* 82: 269-272, 1999.

Austen KF, Boyce JA. Mast cell lineage development and phenotypic regulation. *Leuk Res* 25: 511-518, 2001.

Bach FH, Hancock WW, Ferran C. Protective genes expressed in endothelial cells: a regulatory response to injury. *Immunol Today* 18: 483-486, 1997.

Bachert C. The role of histamine in allergic disease: re-appraisal of its inflammatory potential. *Allergy* 57: 287-296, 2002.

Banovac K, De Forteza R. The effect of mast cell chymase on extracellular matrix: studies in autoimmune thyroiditis and in cultured thyroid cells. *Int Arch Allergy Immunol* 99: 141-149, 1992.

Baram D, Vaday GG, Salamon P, Drucker I, Hershkoviz R, Mekori YA. Human mast cells release metalloproteinase-9 on contact with activated T cells: juxtacrine regulation by TNF-alpha. *J Immunol* 167: 4008-4016, 2001.

Bartold PM, Page RC. A microdetermination method for assaying glycosaminoglycans and proteoglycans. *Anal Biochem* 150: 320-324, 1985.

Bauriedel G, Hutter R, Welsch U, Bach R, Sievert H, Lüderitz B. Role of smooth muscle cell death in advanced coronary primary lesions: implications for plaque instability. *Cardiovasc Res* 41: 480-488, 1999.

Bayer IM, Adamson SL, Langille BL. Atrophic remodeling of the artery-cuffed artery. *Arterioscler Thromb Vasc Biol* 19: 1499-1505, 1999.

Bennett MR. Apoptosis in the cardiovascular system. *Heart* 87: 480-487, 2002.

Bennett MR, Evan GI, Schwartz SM. Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. *J Clin Invest* 95: 2266-2274, 1995.

Benoist C, Mathis D. Mast cells in autoimmune disease. *Nature* 420: 875-878, 2002.

Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation* 91: 2488-2496, 1995.

Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 4: 197-250, 1993.

Björkerud S, Björkerud B. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am J Pathol* 149: 367-380, 1996.

Bobik A, Agrotis A, Kanellakis P, Dilley R, Krushinsky A, Smirnov V, Tararak E, Condron M, Kostolias G. Distinct patterns of transforming growth factor-beta isoform and receptor expression in human atherosclerotic lesions. Colocalization implicates TGF-beta in fibrofatty lesion development. *Circulation* 99: 2883-2891, 1999.

Bombeli T, Karsan A, Tait JF, Harlan JM. Apoptotic vascular endothelial cells become procoagulant. *Blood* 89: 2429-2442, 1997.

Boyle JJ, Weissberg PL, Bennett MR. Human macrophage-induced vascular smooth muscle cell apoptosis requires NO enhancement of Fas/Fas-L interactions. *Arterioscler Thromb Vasc Biol* 22: 1624-1630, 2002.

Bradding P. Human mast cell cytokines. *Clin Exp Allergy* 26: 13-19, 1996.

Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 15: 269-290, 1999.

Burns AR, Bowden RA, Abe Y, Walker DC, Simon SI, Entman ML, Smith CW. P-selectin mediates neutrophil adhesion to endothelial cell borders. *J Leukoc Biol* 65: 299-306, 1999.

Castellot JJ, Jr., Pukac LA, Caleb BL, Wright TC, Jr., Karnovsky MJ. Heparin selectively inhibits a protein kinase C-dependent mechanism of cell cycle progression in calf aortic smooth muscle cells. *J Cell Biol* 109: 3147-3155, 1989.

Chandler JM, Cohen GM, MacFarlane M. Different subcellular distribution of caspase-3 and caspase-7 following Fas-induced apoptosis in mouse liver. *J Biol Chem* 273: 10815-10818, 1998.

Chen M, Wang J. Initiator caspases in apoptosis signaling pathways. *Apoptosis* 7: 313-319, 2002.

Cho A, Courtman DW, Langille BL. Apoptosis (programmed cell death) in arteries of the neonatal lamb. *Circ Res* 76: 168-175, 1995.

Cho A, Mitchell L, Koopmans D, Langille BL. Effects of changes in blood flow rate on cell death and cell proliferation in carotid arteries of immature rabbits. *Circ Res* 81: 328-337, 1997.

Choy JC, Granville DJ, Hunt DW, McManus BM. Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol* 33: 1673-1690, 2001.

Cohen GM. Caspases: the executioners of apoptosis. *Biochem J* 326 (Pt 1): 1-16, 1997.

Compton SJ, Cairns JA, Holgate ST, Walls AF. Human mast cell tryptase stimulates the release of an IL-8-dependent neutrophil chemotactic activity from human umbilical vein endothelial cells (HUVEC). *Clin Exp Immunol* 121: 31-36, 2000.

Cook JW, Taylor LM, Orloff SL, Landry GJ, Moneta GL, Porter JM. Homocysteine and arterial disease. Experimental mechanisms. *Vascul Pharmacol* 38: 293-300, 2002.

Crisby M, Kallin B, Thyberg J, Zhivotovsky B, Orrenius S, Kostulas V, Nilsson J. Cell death in human atherosclerotic plaques involves both oncosis and apoptosis. *Atherosclerosis* 130: 17-27, 1997.

Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci U S A* 87: 5134-5138, 1990.

Cybulsky MI, Gimbrone MA, Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* 251: 788-791, 1991.

D'Angelo M, Billings PC, Pacifici M, Leboy PS, Kirsch T. Authentic matrix vesicles contain active metalloproteases (MMP). a role for matrix vesicle-associated MMP-13 in activation of transforming growth factor-beta. *J Biol Chem* 276: 11347-11353, 2001.

Davies MJ. A macro and micro view of coronary vascular insult in ischemic heart disease. *Circulation* 82: II-38-II-46, 1990.

de Moissac D, Zheng H, Kirshenbaum LA. Linkage of the BH4 domain of Bcl-2 and the nuclear factor kappaB signaling pathway for suppression of apoptosis. *J Biol Chem* 274: 29505-29509, 1999.

Di Girolamo N, Wakefield D. In vitro and in vivo expression of interstitial collagenase/MMP-1 by human mast cells. *Dev Immunol* 7: 131-142, 2000.

Dimmeler S, Haendeler J, Galle J, Zeiher AM. Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases. A mechanistic clue to the 'response to injury' hypothesis. *Circulation* 95: 1760-1763, 1997.

Dimmeler S, Haendeler J, Rippmann V, Nehls M, Zeiher AM. Shear stress inhibits apoptosis of human endothelial cells. *FEBS Lett* 399: 71-74, 1996.

Dimmeler S, Haendeler J, Zeiher AM. Regulation of endothelial cell apoptosis in atherothrombosis. *Curr Opin Lipidol* 13: 531-536, 2002.

Dimmeler S, Hermann C, Zeiher AM. Apoptosis of endothelial cells. Contribution to the pathophysiology of atherosclerosis? *Eur Cytokine Netw* 9: 697-698, 1998.

Dvorak AM, Schleimer RP, Schulman ES, Lichtenstein LM. Human mast cells use conservation and condensation mechanisms during recovery from degranulation. In vitro studies with mast cells purified from human lungs. *Lab Invest* 54: 663-678, 1986.

Dypbukt JM, Ankarcrona M, Burkitt M, Sjoholm A, Strom K, Orrenius S, Nicotera P. Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines. *J Biol Chem* 269: 30553-30560, 1994.

Ehrlich P. Beiträge zur Kenntniss der granulirten Bindegewebszellen und der eosinophilen Leukocythen. *Arch Anat Physiol* 3: 166-169, 1879.

Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ* 5: 551-562, 1998.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 148: 2207-2216, 1992.

Falk E. Why do plaques rupture? *Circulation* 86 (suppl III): 30-42, 1992.

Fan J, Watanabe T. Inflammatory reactions in the pathogenesis of atherosclerosis. *J Atheroscler Thromb* 10: 63-71, 2003.

Farb A, Burke AP, Tang AL, Liang TY, Mannan P, Smialek J, Virmani R. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. *Circulation* 93: 1354-1363, 1996.

Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* 134: 793-799, 1996.

Fuster V. Lewis A. Conner Memorial Lecture. Mechanisms leading to myocardial infarction: insights from studies of vascular biology. *Circulation* 90: 2126-2146, 1994.

Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes. *N Engl J Med* 326: 242-250, 1992.

Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 94: 2493-2503, 1994.

Gallagher SJ, Marshall JS, Hoskin DW. Human mast cells induce caspase-independent DNA fragmentation in leukemic T cells. *Oncol Rep* 10: 1019-1023, 2003.

Galli SJ. New insight into 'the riddle of the mast cells': microenvironmental regulation of mast cell development and phenotypic heterogeneity. *Lab Invest* 62: 5-33, 1990.

Galli SJ, Iemura A, Garlick DS, Gamba-Vitalo C, Zsebo KM, Andrews RG. Reversible expansion of primate mast cell populations in vivo by stem cell factor. *J Clin Invest* 91: 148-152, 1993.

Geng Y, Libby P. Evidence for apoptosis in advanced human atheroma: colocalization with interleukin-1β-converting enzyme. *Am J Pathol* 147: 251-266, 1995.

Geng YJ, Azuma T, Tang JX, Hartwig JH, Muszynski M, Wu Q, Libby P, Kwiatkowski DJ. Caspase-3-induced gelsolin fragmentation contributes to actin cytoskeletal collapse, nucleolysis, and apoptosis of vascular smooth muscle cells exposed to proinflammatory cytokines. *Eur J Cell Biol* 77: 294-302, 1998.

Geng YJ, Henderson LE, Levesque EB, Muszynski M, Libby P. Fas is expressed in human atherosclerotic intima and promotes apoptosis of cytokine-primed human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 17: 2200-2208, 1997.

Geng YJ, Libby P. Progression of atheroma: a struggle between death and procreation. *Arterioscler Thromb Vasc Biol* 22: 1370-1380, 2002.

Gerondakis S, Grossmann M, Nakamura Y, Pohl T, Grumont R. Genetic approaches in mice to understand Rel/NF-kappaB and IkappaB function: transgenics and knockouts. *Oncogene* 18: 6888-6895, 1999.

Giancotti FG, Ruoslahti E. Integrin signaling. *Science* 285: 1028-1032, 1999.

Gordon JR. TGFbeta1 and TNFalpha secreted by mast cells stimulated via the FcepsilonRI activate fibroblasts for high-level production of monocyte chemoattractant protein-1 (MCP-1). *Cell Immunol* 201: 42-49, 2000.

Gordon JR, Galli SJ. Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. *J Exp Med* 174: 103-107, 1991.

Gruber BL, Marchese MJ, Suzuki K, Schwartz LB, Okada Y, Nagase H, Ramamurthy NS. Synovial procollagenase activation by human mast cell tryptase dependence upon matrix metalloproteinase 3 activation. *J Clin Invest* 84: 1657-1662, 1989.

Haley KJ, Lilly CM, Yang JH, Feng Y, Kennedy SP, Turi TG, Thompson JF, Sukhova GH, Libby P, Lee RT. Overexpression of eotaxin and the CCR3 receptor in human atherosclerosis: using genomic technology to identify a potential novel pathway of vascular inflammation. *Circulation* 102: 2185-2189, 2000.

Han DKM, Haudenschild CC, Hong MK, Tinkle BT, Leon MB, Liau G. Evidence for apoptosis in human atherogenesis and in a rat vascular injury model. *Am J Pathol* 147: 267-277, 1995.

Hara M, Matsumori A, Ono K, Kido H, Hwang MW, Miyamoto T, Iwasaki A, Okada M, Nakatani K, Sasayama S. Mast cells cause apoptosis of cardiomyocytes and proliferation of other intramyocardial cells in vitro. *Circulation* 100: 1443-1449, 1999.

Harker LA, Schwartz SM, Ross R. Endothelium and arteriosclerosis. *Clin Haematol* 10: 283-296, 1981.

He S, Peng Q, Walls AF. Potent induction of a neutrophil and eosinophil-rich infiltrate in vivo by human mast cell tryptase: selective enhancement of eosinophil recruitment by histamine. *J Immunol* 159: 6216-6225, 1997.

He S, Walls AF. Human mast cell chymase induces the accumulation of neutrophils, eosinophils and other inflammatory cells in vivo. *Br J Pharmacol* 125: 1491-1500, 1998.

Hedin UL, Daum G, Clowes AW. Disruption of integrin alpha 5 beta 1 signaling does not impair PDGF-BB- mediated stimulation of the extracellular signal-regulated kinase pathway in smooth muscle cells. *J Cell Physiol* 172: 109-116, 1997.

Hedman K, Kurkinen M, Alitalo K, Vaheri A, Johansson S, Höök M. Isolation of the pericellular matrix of human fibroblast cultures. *J Cell Biol* 81: 83-91, 1979.

Hegyi L, Skepper JN, Cary NRB, Mitchinson MJ. Foam cell apoptosis and the development of the lipid core of human atherosclerosis. *J Pathol* 180: 423-429, 1996.

Henderson EL, Geng YJ, Sukhova GK, Whittemore AD, Knox J, Libby P. Death of smooth muscle cells and expression of mediators of apoptosis by T lymphocytes in human abdominal aortic aneurysms. *Circulation* 99: 96-104, 1999.

Hengartner MO. The biochemistry of apoptosis. *Nature* 407: 770-776, 2000.

Ho FM, Liu SH, Liau CS, Huang PJ, Lin-Shiau SY. High glucose-induced apoptosis in human endothelial cells is mediated by sequential activations of c-Jun NH(2)-terminal kinase and caspase-3. *Circulation* 101: 2618-2624, 2000.

Holmes DR, Lopez-Candales A, Liao S, Thompson RW. Smooth muscle cell apoptosis and p53 expression in human abdominal aortic aneurysms. *Ann N Y Acad Sci* 800: 286-287, 1996.

Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proc Natl Acad Sci U S A* 83: 4464-4468, 1986.

Ishizaka T, Ishizaka K. Activation of mast cells for mediator release through IgE receptors. *Prog Allergy* 34: 188-235, 1984.

Isner JM, Kearney M, Bortman S, Passeri J. Apoptosis in human atherosclerosis and restenosis. *Circulation* 91: 2703-2711, 1995.

Jeziorska M, McCollum C, Woolley DE. Mast cell distribution, activation, and phenotype in atherosclerotic lesions of human carotid arteries. *J Pathol* 182: 115-122, 1997.

Johnson JL, Jackson CL, Angelini GD, George SJ. Activation of matrix-degrading metalloproteinases by mast cell proteases in atherosclerotic plaques. *Arterioscler Thromb Vasc Biol*1707-1715, 1998.

Järveläinen H, Wight TN. Vascular Proteoglycans. Garg HG, Roughley PJ, and Hales CA (eds) Proteoglycans in lung disease, p. 291-321 Marcel Dekker, New York, 2002.

Kaartinen M, Penttilä A, Kovanen PT. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation* 90: 1669-1678, 1994a.

Kaartinen M, Penttilä A, Kovanen PT. Mast cells of two types differing in neutral protease composition in the human aortic intima. Demonstration of both tryptase- and chymase-containing mast cells in normal intimas, fatty streaks, and the shoulder region of atheromas. *Arterioscler Thromb* 14: 966-972, 1994b.

Kaartinen M, Penttilä A, Kovanen PT. Mast cells in rupture-prone areas of human coronary atheromas produce and store TNF-α. *Circulation* 94: 2787-2792, 1996.

Kaartinen M, van der Wal AC, van der Loos CM, Piek JJ, Koch KT, Becker AE, Kovanen PT. Mast cell infiltration in acute coronary syndromes: implications for plaque rupture. *J Am Coll Cardiol* 32: 606-612, 1998.

Kam CM, Hudig D, Powers JC. Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors. *Biochim Biophys Acta* 1477: 307-323, 2000.

Karsan A, Yee E, Harlan JM. Endothelial cell death induced by tumor necrosis factor-alpha is inhibited by the Bcl-2 family member, A1. *J Biol Chem* 271: 27201-27204, 1996.

Katz HR, Austen KF, Caterson B, Stevens RL. Secretory granules of heparin-containing rat serosal mast cells also possess highly sulfated chondroitin sulfate proteoglycans. *J Biol Chem* 261: 13393-13396, 1986.

Kauhanen P, Kovanen PT, Lassila R. Coimmobilized native macromolecular heparin proteoglycans strongly inhibit platelet-collagen interactions in flowing blood. *Arterioscler Thromb Vasc Biol* 20: E113-E119, 2000.

Kawasaki M, Kuwano K, Hagimoto N, Matsuba T, Kunitake R, Tanaka T, Maeyama T, Hara N. Protection from lethal apoptosis in lipopolysaccharide-induced acute lung injury in mice by a caspase inhibitor. *Am J Pathol* 157: 597-603, 2000.

Kelley JL, Chi DS, Abou-Auda W, Smith JK, Krishnaswamy G. The molecular role of mast cells in atherosclerotic cardiovascular disease. *Mol Med Today* 6: 304-308, 2000.

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26: 239-257, 1972.

Kilpatrick LE, Lee JY, Haines KM, Campbell DE, Sullivan KE, Korchak HM. A role for PKC-delta and PI 3-kinase in TNF-alpha-mediated antiapoptotic signaling in the human neutrophil. *Am J Physiol Cell Physiol* 283: C48-C57, 2002.

Kim HS, Hwang KK, Seo JW, Kim SY, Oh BH, Lee MM, Park YB. Apoptosis and regulation of Bax and Bcl-X proteins during human neonatal vascular remodeling. *Arterioscler Thromb Vasc Biol* 20: 957-963, 2000.

Kitamura Y. Heterogeneity of mast cells and phenotypic change between subpopulations. *Ann Rev Immunol* 7: 59-76, 1989.

Kitamura Y, Go S. Decreased production of mast cells in S1/S1d anemic mice. *Blood* 53: 492-497, 1979.

Kitamura Y, Go S, Hatanaka K. Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. *Blood* 52: 447-452, 1978.

Kockx MM, De Meyer GRY, Muhring J, Jacob W, Bult H, Herman AG. Apoptosis and related proteins in different stages of human atherosclerotic plaques. *Circulation* 97: 2307-2315, 1998.

Kockx MM, Herman AG. Apoptosis in atherosclerosis: beneficial or detrimental? *Cardiovasc Res* 45: 736-746, 2000.

Kokkonen JO, Kovanen PT. Low density lipoprotein degradation by rat mast cells: Demonstration of extracellular proteolysis caused by mast cell granules. *J Biol Chem* 260: 14756-14763, 1985.

Kokkonen JO, Kovanen PT. Stimulation of mast cells leads to cholesterol accumulation in macrophages in vitro by a mast cell granule-mediated uptake of low density lipoprotein. *Proc Natl Acad Sci U S A* 84: 2287-2291, 1987.

Kokkonen JO, Kovanen PT. Proteolytic enzymes of mast cell granules degrade low density lipoproteins and promote their granule-mediated uptake by macrophages in vitro. *J Biol Chem* 264: 10749-10755, 1989.

Kokkonen JO, Saarinen J, Kovanen PT. Regulation of local angiotensin II formation in the human heart in the presence of interstitial fluid. Inhibition of chymase by protease inhibitors of interstitial fluid and of angiotensin converting enzyme by Ang-(1-9) formed by heart carboxypeptidase A-like activity. *Circulation* 95: 1455-1463, 1997.

Kokkonen JO, Vartiainen M, Kovanen PT. Low density lipoprotein degradation by secretory ganules of rat mast cells. Sequential degradation of apolipoprotein B by granule chymase and carboxypeptidase A. *J Biol Chem* 261: 16067-16072, 1986.

Kolodgie FD, Burke AP, Farb A, Gold HK, Yuan J, Narula J, Finn AV, Virmani R. The thin-cap fibroatheroma: a type of vulnerable plaque: the major precursor lesion to acute coronary syndromes. *Curr Opin Cardiol* 16: 285-292, 2001.

Kondo S, Hashimoto N, Kikuchi H, Hazama F, Nagata I, Kataoka H. Apoptosis of medial smooth muscle cells in the development of saccular cerebral aneurysms in rats. *Stroke* 29: 181-188, 1998.

Koseki C, Herzlinger D, al Awqati Q. Apoptosis in metanephric development. *J Cell Biol* 119: 1327-1333, 1992.

Kovanen PT. Atheroma formation: defective control in the intimal round-trip of cholesterol. *Eur Heart J* 11: 238-246, 1990.

Kovanen PT. The mast cell - a potential link between inflammation and cellular cholesterol deposition in atherogenesis. *Eur Heart J* 14 (Suppl K): 105-117, 1993.

Kovanen PT. Role of mast cells in atherosclerosis. Marone G (ed) Human basophils and mast cells: clinical aspects., p. 132-170 S. Karger, Basel, 1995.

Kovanen PT, Kaartinen M, Paavonen T. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation* 92: 1084-1088, 1995.

Laine P, Kaartinen M, Penttilä A, Panula P, Paavonen T, Kovanen PT. Association between myocardial infarction and the mast cells in the adventitia of the infarct-related coronary artery. *Circulation* 99: 361-369, 1999.

Laine P, Pentikäinen MO, Wurzner R, Penttilä A, Paavonen T, Meri S, Kovanen PT. Evidence for complement activation in ruptured coronary plaques in acute myocardial infarction. *Am J Cardiol* 90: 404-408, 2002.

Lassila R, Lindstedt KA, Kovanen PT. Native macromolecular heparin proteoglycans exocytosed from stimulated rat serosal mast cells strongly inhibit platelet-collagen interactions. *Arterioscler Thromb Vasc Biol* 17: 3578-3587, 1997.

Lassus P, Opitz-Araya X, Lazebnik Y. Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 297: 1352-1354, 2002.

Lee M, Lindstedt LK, Kovanen PT. Mast cell-mediated inhibition of reverse cholesterol transport. *Arterioscler Thromb* 12: 1329-1335, 1992.

Lee M, Sommerhoff CP, von Eckardstein A, Zettl F, Fritz H, Kovanen PT. Mast cell tryptase degrades HDL and blocks its function as an acceptor of cellular cholesterol. *Arterioscler Thromb Vasc Biol* 22: 2086-2091, 2002.

Lee M, von Eckardstein A, Lindstedt L, Assmann G, Kovanen PT. Depletion of pre beta 1LpA1 and LpA4 particles by mast cell chymase reduces cholesterol efflux from macrophage foam cells induced by plasma. *Arterioscler Thromb Vasc Biol* 19: 1066-1074, 1999.

Lee RT, Libby P. The unstable atheroma. *Arterioscler Thromb Vasc Biol* 17: 1859-1867, 1997.

Leszczynski D. Regulation of cell cycle and apoptosis by protein kinase C in rat myeloid leukemia cell line. *Oncology Res* 7: 471-480, 1995.

Leszczynski D, Zhao Y, Luokkamäki M, Foegh ML. Apoptosis of vascular smooth muscle cells. Protein kinase C and oncoprotein Bcl-2 are involved in regulation of apoptosis in non-transformed rat vascular smooth muscle cells. *Am J Pathol* 145: 1265-1270, 1994.

Li H, Cybulsky MI, Gimbrone MA, Jr., Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *Arterioscler Thromb* 13: 197-204, 1993a.

Li H, Cybulsky MI, Gimbrone MA, Jr., Libby P. Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. *Am J Pathol* 143: 1551-1559, 1993b.

Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94: 491-501, 1998.

Li L, Krilis SA. Mast-cell growth and differentiation. *Allergy* 54: 306-312, 1999.

Libby P. Inflammation in atherosclerosis. *Nature* 420: 868-874, 2002.

Lijnen HR. Extracellular proteolysis in the development and progression of atherosclerosis. *Biochem Soc Trans* 30: 163-167, 2002.

Lindstedt KA, Kokkonen JO, Kovanen PT. Soluble heparin proteoglycans released from stimulated mast cells induce uptake of low density lipoproteins by macrophages via scavenger receptor-mediated phagocytosis. *J Lipid Res* 33: 65-75, 1992.

Lindstedt KA, Kokkonen JO, Kovanen PT. Inhibition of copper-mediated oxidation of LDL by rat serosal mast cells: A novel cellular protective mechanism involving proteolysis of the substrate under oxidative stress. *Arterioscler Thromb* 13: 23-32, 1993.

Lindstedt KA, Kokkonen JO, Kovanen PT. Regulation of the activity of secreted human lung mast cell tryptase by mast cell proteoglycans. *Biochim Biophys Acta* 1425: 617-627, 1998.

Lindstedt KA, Wang Y, Shiota N, Saarinen J, Hyytiäinen M, Kokkonen JO, Keski-Oja J, Kovanen PT. Activation of paracrine TGF-beta1 signaling upon stimulation and degranulation of rat serosal mast cells: a novel function for chymase. *FASEB J* 15: 1377-1388, 2001b.

Lindstedt L, Lee M, Castro GR, Fruchart J, Kovanen PT. Chymase in exocytosed rat mast cell granules effectively proteolyzes apolipoprotein A1-containing lipoproteins, so reducing the cholesterol efflux-inducing ability of serum and aortic intimal fluid. *J Clin Invest* 97: 2174-2182, 1996.

Lindstedt L, Lee M, Kovanen PT. Chymase bound to heparin is resistant to its natural inhibitors and capable of proteolyzing high density lipoproteins in aortic intimal fluid. *Atherosclerosis* 155: 87-97, 2001a.

Liu MC, Proud D, Lichtenstein LM, MacGlashan DW, Schleimer RP, Adkinson NF, Kagey-Sobotka A, Schulman ES, Plaut M. Human lung macrophage-derived histamine-releasing activity is due to IgE-dependent factors. *J Immunol* 136: 2588-2595, 1986.

Lohi J, Harvima I, Keski-Oja J. Pericellular substrates of human mast cell tryptase: 72,000 dalton gelatinase and fibronectin. *J Cell Biochem* 50: 337-349, 1992.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.

Lutgens E, Gijbels M, Smook M, Heeringa P, Gotwals P, Koteliansky VE, Daemen MJ. Transforming growth factor-beta mediates balance between inflammation and fibrosis during plaque progression.

Arterioscler Thromb Vasc Biol 22: 975-982, 2002.

Ma H, Kovanen PT. Degranulation of cutaneous mast cells induces transendothelial transport and local accumulation of plasma LDL in rat skin in vivo. *J Lipid Res* 38: 1877-1887, 1997.

Madden KB, Urban JF, Jr., Ziltener HJ, Schrader JW, Finkelman FD, Katona IM. Antibodies to IL-3 and IL-4 suppress helminth-induced intestinal mastocytosis. *J Immunol* 147: 1387-1391, 1991.

Maier M, Spragg J, Schwartz LB. Inactivation of human high molecular weight kininogen by human mast cell tryptase. *J Immunol* 130: 2352-2356, 1983.

Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. *Circulation* 99: 348-353, 1999.

Mallat Z, Tedgui A. Current perspective on the role of apoptosis in atherothrombotic disease. *Circ Res* 88: 998-1003, 2001.

Matter ML, Ruoslahti E. A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription. *J Biol Chem* 276: 27757-27763, 2001.

Matter ML, Zhang Z, Nordstedt C, Ruoslahti E. The alpha5beta1 integrin mediates elimination of amyloid-beta peptide and protects against apoptosis. *J Cell Biol* 141: 1019-1030, 1998.

Mayer B, Oberbauer R. Mitochondrial regulation of apoptosis. *News Physiol Sci* 18:89-94.: 89-94, 2003.

Mayr M, Xu Q. Smooth muscle cell apoptosis in arteriosclerosis. *Exp Gerontol* 36: 969-987, 2001.

McGill HC, Jr. Fatty streaks in the coronary arteries and aorta. *Lab Invest* 18: 560-564, 1968.

Mekori YA, Metcalfe DD. Mast cells in innate immunity. *Immunol Rev* 173: 131-140, 2000.

Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev* 77: 1033-1079, 1997.

Metcalfe DD, Kaliner M, Donlon MA. The mast cell. *Crit Rev Immunol* 3: 23-74, 1981.

Molin DG, DeRuiter MC, Wisse LJ, Azhar M, Doetschman T, Poelmann RE, Gittenberger-de Groot AC. Altered apoptosis pattern during pharyngeal arch artery remodelling is associated with aortic arch malformations in Tgfbeta2 knock-out mice. *Cardiovasc Res* 56: 312-322, 2002.

Moreno PR, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT. Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. *Circulation* 90: 775-778, 1994.

Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett* 82: 85-91, 2002.

Munro JM, Cotran RS. Biology of Disease. The pathogenesis of atherosclerosis: atherogenesis and inflammation. *Lab Invest* 58: 249-261, 1988.

Murata K, Motayama T, Kotake C. Collagen types in various layers of the human aorta and their changes with the atherosclerotic process. *Atherosclerosis* 60: 251-262, 1986.

Murray,CJL, A d Lopez. The Global Burden of Disease. A comprehensive assessment of mortality and disability from diseases, injuries and risk factors in 1990 and projected to 2020. Harvard School of Public Health on behalf of the World Health Organisation and the World Bank Cambridge, Massachusetts 1996.

Müller JM, Ziegler-Heitbrock HW, Baeuerle PA. Nuclear factor kappa B, a mediator of lipopolysaccharide effects. *Immunobiology* 187: 233-256, 1993.

Napoli C, D'Armiento FP, Mancini FP, Postiglione A, Witztum JL, Palumbo G, Palinski w. Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. *J Clin Invest* 100: 2680-2690, 1997.

Naruko T, Ueda M, Haze K, van der Wal AC, van der Loos CM, Itoh A, Komatsu R, Ikura Y, Ogami M, Shimada Y, Ehara S, Yoshiyama M, Takeuchi K, Yoshikawa J, Becker AE. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* 106: 2894-2900, 2002.

Nelimarkka LO, Nikkari ST, Ravanti LS, Kähäri VM, Järvelainen HT. Collagenase-1, stromelysin-1 and 92 kDa gelatinase are associated with tumor necrosis factor-alpha induced morphological change of human endothelial cells in vitro. *Matrix Biol* 17: 293-304, 1998.

Niemann-Jonsson A, Ares MP, Yan ZQ, Bu DX, Fredrikson GN, Branen L, Porn-Ares I, Nilsson AH, Nilsson J. Increased rate of apoptosis in intimal arterial smooth muscle cells through endogenous activation of TNF receptors. *Arterioscler Thromb Vasc Biol* 21: 1909-1914, 2001.

Nikkari ST, O´Brien KD, Ferguson M, Hatsukami T, Welgus HG, Alpers CE, Clowes AW. Interstitial collagenase (MMP-1) expression in human carotid atherosclerosis. *Circulation* 92: 1393-1398, 1995.

O'Brien V, Frisch SM, Juliano RL. Expression of the integrin alpha 5 subunit in HT29 colon carcinoma cells suppresses apoptosis triggered by serum deprivation. *Exp Cell Res* 224: 208-213, 1996.

Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18: 6853-6866, 1999.

Pejler G, Berg L. Regulation of rat mast cell protease 1 activity . Protease inhibition is prevented by heparin proteoglycan. *Eur J Biochem* 233: 192-199, 1995.

Pentikäinen MO, Öörni K, Ala-Korpela M, Kovanen PT. Modified LDL - trigger of atherosclerosis and inflammation in the arterial intima. *J Intern Med* 247: 359-370, 2000.

Pentikäinen V, Suomalainen L, Erkkilä K, Martelin E, Parvinen M, Pentikäinen MO, Dunkel L. Nuclear factor-kappa B activation in human testicular apoptosis. *Am J Pathol* 160: 205-218, 2002.

Perlman H, Maillard L, Krasinski K, Walsh K. Evidence for the rapid onset of apoptosis in medial smooth muscle cells after balloon injury. *Circulation* 95: 981-987, 1997.

Perlman H, Pagliari LJ, Georganas C, Mano T, Walsh K, Pope RM. FLICE-inhibitory protein expression during macrophage differentiation confers resistance to fas-mediated apoptosis. *J Exp Med* 190: 1679-1688, 1999.

Pickering JG, Weir L, Jekanowski J, Kearney MA, Isner JM. Proliferative activity in peripheral and coronary atherosclerotic plaque among patients undergoing percutaneous revascularization. *J Clin Invest* 91: 1469-1480, 1993.

Piha M, Lindstedt L, Kovanen PT. Fusion of proteolyzed low-density lipoprotein in the fluid phase: a novel mechanism generating atherogenic lipoprotein particles. *Biochemistry* 34: 10120-10129, 1995.

Piper HM, Spahr R, Mertens S, Krützfeldt A, Watanabe H. Microvascular endothelial cells from heart. Piper HM (ed) Cell culture techniques in heart and vessel research, p. 158-177 Springer Verlag, Berlin, 1990.

Pober JS, Gimbrone MA, Jr., Lapierre LA, Mendrick DL, Fiers W, Rothlein R, Springer TA. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J Immunol* 137: 1893-1896, 1986.

Pohlman TH, Harlan JM. Human endothelial cell response to lipopolysaccharide, interleukin-1, and tumor necrosis factor is regulated by protein synthesis. *Cell Immunol* 119: 41-52, 1989.

Polunovsky VA, Chen B, Henke C, Snover D, Wendt C, Ingbar DH, Bitterman PB. Role of mesenchymal cell death in lung remodeling after injury. *J Clin Invest* 92: 388-397, 1993.

Polunovsky VA, Wendt CH, Ingbar DH, Peterson MS, Bitterman PB. Induction of endothelial cell apoptosis by TNF alpha: modulation by inhibitors of protein synthesis. *Exp Cell Res* 214: 584-594, 1994.

Puxeddu I, Piliponsky AM, Bachelet I, Levi-Schaffer F. Mast cells in allergy and beyond. *Int J Biochem Cell Biol* 35: 1601-1607, 2003.

Ra C, Yasuda M, Yagita H, Okumura K. Fibronectin receptor integrins are involved in mast cell activation. *J Allergy Clin Immunol* 94: 625-628, 1994.

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* 262: 695-700, 1993.

Rath PC, Aggarwal BB. TNF-induced signaling in apoptosis. *J Clin Immunol* 19: 350-364, 1999.

Repka-Ramirez MS, Baraniuk JN. Histamine in health and disease. *Clin Allergy Immunol* 17:1-25.: 1-25, 2002.

Richardson PD, Davies MJ, Born GVR. Influence of plaque configuration and stress distribution on fissuring of coronary atherosclerotic plaque. *Lancet* 2: 941-944, 1989.

Rivard A, Luo Z, Perlman H, Fabre JE, Nguyen T, Maillard L, Walsh K. Early cell loss after angioplasty results in a disproportionate decrease in percutaneous gene transfer to the vessel wall. *Hum Gene Ther* 10: 711-721, 1999.

Rodewald HR, Dessing M, Dvorak AM, Galli SJ. Identification of a committed precursor for the mast cell lineage. *Science* 271: 818-822, 1996.

Rohlich P, Anderson P, Uvnas B. Electron microscope observations on compounds 48-80-induced degranulation in rat mast cells. Evidence for sequential exocytosis of storage granules. *J Cell Biol* 51: 465-483, 1971.

Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med* 340: 115-126, 1999.

Ross R, Glomset JA. The pathogenesis of atherosclerosis. *N Engl J Med* 295: 369-377-420-425, 1976.

Rossig L, Haendeler J, Hermann C, Malchow P, Urbich C, Zeiher AM, Dimmeler S. Nitric oxide down-regulates MKP-3 mRNA levels: involvement in endothelial cell protection from apoptosis. *J Biol Chem* 275: 25502-25507, 2000.

Saarinen J, Kalkkinen N, Welgus HG, Kovanen PT. Activation of human interstitial procollagenase through direct cleavage of the Leu^{83}-Thr^{84} bond by mast cell chymase. *J Biol Chem* 269: 18134-18140, 1994.

Saikku P. Chlamydia pneumoniae in atherosclerosis. *J Intern Med* 247: 391-396, 2000.

Saiura A, Sata M, Hirata Y, Nagai R, Makuuchi M. Circulating smooth muscle progenitor cells contribute to atherosclerosis. *Nat Med* 7: 382-383, 2001.

Saren P, Welgus HG, Kovanen PT. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. *J Immunol* 157: 4159-4165, 1996.

Scatena M, Almeida M, Chaisson ML, Fausto N, Nicosia RF, Giachelli CM. NF-kappaB mediates alphavbeta3 integrin-induced endothelial cell survival. *J Cell Biol* 141: 1083-1093, 1998.

Schechter NM, Irani AM, Sprows JL, Abernethy J, Wintroub B, Schwartz LB. Identification of a cathepsin G-like proteinase in the MCTC type of human mast cell. *J Immunol* 145: 2652-2661, 1990.

Scheid MP, Woodgett JR. Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett* 546: 108-112, 2003.

Schwartz LB, Austen KF. Structure and Function of the Chemical Mediators of Mast Cells. Ishizaka K (ed) Mast cell activation and mediator release, p. 271-321 S. Karger, Basel, 1984.

Schwartz LB, Bradford TR. Regulation of tryptase from human lung mast cells by heparin. Stabilization of the active tetramer. *J Biol Chem* 261: 7372-7379, 1986.

Schwartz LB, Bradford TR, Littman BH, Wintroub BU. The fibrinogenolytic activity of purified tryptase from human lung mast cells. *J Immunol* 135: 2762-2767, 1985.

Schweizer A, Briand C, Grutter MG. Crystal structure of caspase-2, apical initiator of the intrinsic apoptotic pathway. *J Biol Chem* .: epub ahead of print, 2003.

Sedgwick JD, Holt PG, Turner KJ. Production of a histamine-releasing lymphokine by antigen- or mitogen-stimulated human peripheral T cells. *Clin Exp Immunol* 45: 409-418, 1981.

Seshiah PN, Kereiakes DJ, Vasudevan SS, Lopes N, Su BY, Flavahan NA, Goldschmidt-Clermont PJ. Activated monocytes induce smooth muscle cell death: role of macrophage colony-stimulating factor and cell contact. *Circulation* 105: 174-180, 2002.

Slee EA, Zhu H, Chow SC, MacFarlane M, Nicholson DW, Cohen GM. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK) inhibits apoptosis by blocking the processing of CPP32. *Biochem J* 315: 21-24, 1996.

Slomp J, Gittenberger-de GA, Glukhova MA, Conny van Munsteren J, Kockx MM, Schwartz SM, Koteliansky VE. Differentiation, dedifferentiation, and apoptosis of smooth muscle cells during the development of the human ductus arteriosus. *Arterioscler Thromb Vasc Biol*

17: 1003-1009, 1997.

Slowik MR, Min W, Ardito T, Karsan A, Kashgarian M, Pober JS. Evidence that tumor necrosis factor triggers apoptosis in human endothelial cells by interleukin-1-converting enzyme-like protease- dependent and -independent pathways. *Lab Invest* 77: 257-267, 1997.

Smith TJ, Hougland MW, Johnson DA. Human lung tryptase. Purification and characterization. *J Biol Chem* 259: 11046-11051, 1984.

Stary HC. The sequence of cell and matrix changes in atherosclerotic lesions of coronary arteries in the first forty years of life. *Eur Heart J* 11(suppl. E): 3-19, 1990.

Stary HC. Lipid and macrophage accumulations in arteries of children and the development of atherosclerosis. *Am J Clin Nutr* 72: 1297S-1306S, 2000.

Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 92: 1355-1374, 1995.

Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W, Jr., Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb* 14: 840-856, 1994.

Stender S, Zilversmit DB. Transfer of plasma lipoprotein components and of plasma proteins into aortas of cholesterol-fed rabbits. Molecular size as a determinant of plasma lipoprotein influx. *Arteriosclerosis* 1: 38-49, 1981.

Stevens RL, Fox CC, Lichtenstein LM, Austen KF. Identification of chondroitin sulfate E proteoglycans and heparin proteoglycans in the secretory granules of human lung mast cells. *Proc Natl Acad Sci U S A* 85: 2284-2287, 1988.

Strauss WM. Preparation of genomic DNA from mammalian tissue. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K (eds) Current protocols in molecular biology, p. 2.2.1-2.2.3 John Wiley & Sons, New York, 1987.

Stupack DG, Puente XS, Boutsaboualoy S, Storgard CM, Cheresh DA. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J Cell Biol* 155: 459-470, 2001.

Tafani M, Schneider TG, Pastorino JG, Farber JL. Cytochrome c-dependent activation of caspase-3 by tumor necrosis factor requires induction of the mitochondrial permeability transition. *Am J Pathol* 156: 2111-2121, 2000.

Taguchi S, Oinuma T, Yamada T. A comparative study of cultured smooth muscle cell proliferation and injury, utilizing glycated low density lipoproteins with slight oxidation, auto-oxidation, or extensive oxidation. *J Atheroscler Thromb* 7: 132-137, 2000.

Taipale J, Koli K, Keski-Oja J. Release of transforming growth factor-β1 from the pericellular matrix of cultured fibroblasts and fibrosarcoma cells by plasmin and thrombin. *J Biol Chem* 267: 25378-25384, 1992.

Taipale J, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-β1 from the extracellular matrix of cultured human epithelial and endothelial cells. *J Biol Chem* 270: 4689-4696, 1995.

Taipale J, Miyazono K, Heldin C, Keski-Oja J. Latent transforming growth factor-β1 associates to fibroblast extracellular matrix via latent TGF-β binding protein. *J Cell Biol* 124: 171-181, 1994.

Thompson RW, Liao S, Curci JA. Vascular smooth muscle cell apoptosis in abdominal aortic aneurysms. *Coron Artery Dis* 8: 623-631, 1997.

Trapani JA. Granzymes: a family of lymphocyte granule serine proteases. *Genome Biol* 2: 3014.1-3014.7, 2001.

Travis J, Salvesen GS. Human plasma proteinase inhibitors. *Ann Rev Biochem* 52: 655-709, 1983.

Trevani AS, Andonegui G, Giordano M, Nociari M, Fontán P, Dran G, Geffner JR. Neutrophil apoptosis induced by proteolytic enzymes. *Lab Invest* 74: 711-721, 1996.

Tricot O, Mallat Z, Heymes C, Belmin J, Leseche G, Tedgui A. Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques. *Circulation* 101: 2450-2453, 2000.

Troy CM, Shelanski ML. Caspase-2 redux. *Cell Death Differ* 10: 101-107, 2003.

Valent P, Spanblochl E, Sperr WR, Sillaber C, Zsebo KM, Agis H, Strobl H, Geissler K, Bettelheim P, Lechner K. Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long-term culture. *Blood* 80: 2237-2245, 1992.

van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation* 89: 36-44, 1994.

van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De WB, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. *Circ Res* 88: 780-786, 2001.

Vartio T, Seppä H, Vaheri A. Susceptibility of soluble and matrix fibronectins to degradation by tissue proteinases, mast cell chymase and cathepsin G. *J Biol Chem* 256: 471-477, 1981.

Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 20: 1262-1275, 2000.

Wallach D. Cell death induction by TNF: a matter of self control. *Trends Biochem Sci* 22: 107-109, 1997.

Walsh K, Smith RC, Kim HS. Vascular cell apoptosis in remodeling, restenosis, and plaque rupture. *Circ Res* 87: 184-188, 2000.

Wang J, Wilcken DE, Wang XL. Cigarette smoke activates caspase-3 to induce apoptosis of human umbilical venous endothelial cells. *Mol Genet Metab* 72: 82-88, 2001.

Wang Y, Kovanen PT. Heparin proteoglycans released from rat serosal mast cells inhibit proliferation of rat aortic smooth

muscle cells in culture. *Circ Res* 84: 74-83, 1999.

Wang Y, Lindstedt KA, Kovanen PT. Mast cell granule remnants carry LDL into smooth muscle cells of synthetic phenotype and induce their conversion into foam cells. *Arterioscler Thromb Vasc Biol* 15: 801-810, 1995.

Wasserman SI. Mast cell biology. *J Allergy Clin Immunol* 86: 590-593, 1990.

Weaver VM, Lelievre S, Lakins JN, Chrenek MA, Jones JC, Giancotti F, Werb Z, Bissell MJ. beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. *Cancer Cell* 2: 205-216, 2002.

Wedemeyer J, Tsai M, Galli SJ. Roles of mast cells and basophils in innate and acquired immunity. *Curr Opin Immunol* 12: 624-631, 2000.

Williams CM, Galli SJ. The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. *J Allergy Clin Immunol* 105: 847-859, 2000.

Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* 88: 1785-1792, 1991.

Woodbury RG, Everitt MT, Neurath H. Mast cell proteases. *Methods Enzymol* 80: 588-609, 1981.

Wu NZ, Baldwin AL. Transient venular permeability increase and endothelial gap formation induced by histamine. *Am J Physiol* 262: H1238-H1247, 1992.

Wyllie AH. Apoptosis: an overview. *Br Med Bull* 53: 451-465, 1997.

Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 68: 251-306, 1980.

Yang JJ, Kettritz R, Falk RJ, Jennette JC, Gaido ML. Apoptosis of endothelial cells induced by the neutrophil serine proteases proteinase 3 and elastase. *Am J Pathol* 149: 1617-1626, 1996.

Yang L, Qiu CX, Ludlow A, Ferguson MW, Brunner G. Active transforming growth factor-beta in wound repair: determination using a new assay. *Am J Pathol* 154: 105-111, 1999.

Young JD, Lir CC, Butler G, Cohn ZA, Galli SJ. Identification, purification, and characterization of a mast cell-associated cytolytic factor related to tumor necrosis factor. *Proc Natl Acad Sci U S A* 84: 9157-9159, 1987.

Yurt RW, Leid RW, Jr., Austen KF. Native heparin from rat peritoneal mast cells. *J Biol Chem* 252: 518-521, 1977.